

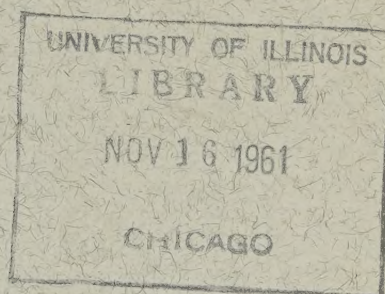
МИКРОБИОЛОГИЯ

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A translation of Mikrobiologiya

(Russian Original Dated March-April, 1961)

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JANUARY PLENUM OF THE CENTRAL COMMITTEE OF THE COMMUNIST PARTY OF THE SOVIET UNION

Translated from *Mikrobiologiya*, Vol. 30, No. 2,
pp. 193-196, March-April, 1961

The Plenum of the C. C. of the C. P. S. U. held in January, 1961, was an outstanding political event and will rightfully occupy a prominent position in the life of the party and the people.

The Plenum adopted a resolution calling for the convocation of the 22nd Congress of the Communist Party of the Soviet Union in October, 1961. At this congress a new party program — the program for the construction of communism — will be discussed and adopted.

The Plenum approved the political line and work of the C. P. S. U. delegation, led by Comrade N. S. Krushchev, at the Conference of the Representatives of Communist and Workers' Parties. This conference, held in November, 1960, was a historical event of worldwide significance.

The question of the state of affairs in the agriculture of the country formed an important part of the work of the January Plenum of the C. C. of the C. P. S. U.

Our party has always given great attention to questions of agricultural development. The primary reason is that this branch of the national economy is of the utmost importance in the life of the people, for the improvement of its welfare, and also for the creation of the material and technical basis of communism. In a resolution, the Plenum noted with satisfaction that our people, under the leadership of the party, are successfully carrying out the magnificent program of communist construction mapped out by the 21st Congress of the C.P.S.U. With the elapse of the first two years of the seven-year plan, considerable advances have been made in the development of industry, science, engineering, culture, and also in the improvement of the material welfare of the workers.

Since the September Plenum of the C. C. of the C. P. S. U., 1953, which was also occupied with agricultural problems, our collective and state farms have made considerable progress.

There has been a great increase in the production and state purchases of all agricultural products. The following data are fairly conclusive evidence of this. In 1953 the grain production was 5 billion, 36 million poods, and in 1960 it was 8 billion, 131 million poods. The meat production in 1953 was 5 million, 822 thousand metric tons (carcass weight), and in 1960 it was 8 million, 725 thousand metric tons. The milk production in 1953 was 36 million, 475 thousand metric tons and in 1960 was 61 million, 538 thousand metric tons.

The quantity of products bought by the state in 1960 was increased over that of 1953; the grain purchase by 1 billion poods, the meat purchase was doubled, and the milk purchase was two and a half times greater.

Despite the unfavorable weather conditions the country produced more grain, sugar beet, oil-producing crops and vegetables in 1960 than in 1959. This enabled the state to sell to the population more meat, milk, butter, sugar, vegetables and other products of the agricultural industry in 1960 than in 1959.

But despite these undoubted achievements our agriculture is still not developing at the same high rate as industry, and is failing to keep pace with the rapid growth of our industry and the increased demand of the population.

The interests of communist construction and the steady improvement in the welfare of the Soviet people now require a higher rate of agricultural development.

Hence, the discussion of agricultural questions by the Plenum was of an exceptionally serious and critical nature. The severity and frankness of the criticism of the shortcomings were evidence of the strength and great authority of our party, of the faith of the party in the unbounded creative power of the people, and in the inexhaustible potentialities of the socialist system. The work of the January Plenum is a splendid example of Bolshevik intransigence to shortcomings and of the constructive tackling of urgent problems.

The Plenum not only exposed the faults in the direction of agriculture, but also pointed out the way to a further improvement in all branches of agriculture.

To agricultural workers the Plenum assigned what was regarded as one of the most important tasks, viz., to secure a steady increase in the supply of agricultural products so that the increasing needs of the population will be fully met and that the supply of these products will always outstrip the demand of the population.

This is a difficult but not impossible task.

The country is now able to make a large increase, more than that envisaged in the seven-year plan, in the capital investment for the development of agriculture and the industry serving agriculture.

The tremendous achievements of our science and technology, the great wealth of experience amassed in the planning and direction of agriculture, and the excellent examples of work by leading agriculturists and innovators make it possible to create conditions in which agriculture will not depend on the caprices of nature.

"Agricultural production," as the Plenum stresses in its resolution, "must be organized in such a way that every year, in any weather conditions, it will ensure the supply of products which the country requires for complete satisfaction of the needs of the people."

Science will play an important part in the tackling of the vitally important problems which the Plenum has assigned to agriculture. In present-day conditions every branch of agriculture depends on science.

The most important and decisive advantage of socialism over capitalism is that under socialism agriculture can be run on a scientific basis, not only in individual farms, but on a country-wide scale.

Our scientists must take an active part in the fulfilment of the state plan for the all-round mechanization of every branch of agriculture.

Scientists will have a large part to play in the development of measures to ensure that in the forthcoming years there will be a transition to continuous methods of cultivation, harvesting, and postharvest treatment of the main agricultural products.

The biological sciences, including microbiology, have great importance as the theoretical bases for agriculture.

An important role in the fulfilment of the tasks which the Plenum has set agricultural science will be played not only by agricultural microbiology but by other sections as well, particularly general and industrial microbiology. The successful resolution of the problems confronting the various sections of microbiology will require a further rise in the level of theoretical studies.

Our microbiologists must draw more and more upon the progress in physics and chemistry and make extensive use of the latest advances in biophysics and biochemistry in their investigations.

There must be an extension of research on the fine structure, physiology, biochemistry, and variation of microorganisms, principally those which are of practical importance. In the resolution of the Plenum, particular attention was given to the utilization of such reserves as increases in the yield of grain and other crops, improvement of tillage, and the provision of an adequate fodder supply. Our scientists working in the field of agricultural microbiology must give special attention to the successful resolution of these questions.

Soil microbiologists must cooperate with other specialists (agronomists, pedologists, agrochemists, etc.) in the development of more effective methods of tilling the soil, preparing, storing, and applying mineral, organomineral, and bacterial fertilizers and introducing them into farming practice.

Soil microbiologists must take an active part in the working out of crop rotations which will ensure that the biochemical activity of soil microorganisms and the products of their activity are fully utilized for raising the crop yield.

Special attention must be given to research on the essential nature of the action of bacterial fertilizers. There must be an improvement in the technology of manufacture of all those bacterial preparations which have proved to be most effective for agriculture.

Our party has successfully carried out the program of reclamation of virgin lands. Forty-one million hectares of new land have been plowed up. The expenditure of the state on reclamation of the virgin land has been fully reimbursed with a large margin. The virgin land is now responsible for 40% of the grain purchase. Of great importance for the virgin regions today is the development of a scientifically based system of soil utilization with due regard to the special features of each zone.

In the fulfilment of this task soil microbiologists will play an important part.

The Plenum noted that irrigation is a reliable means of securing assured harvests. The research plans of our agricultural microbiologists must provide for a study of the effect of irrigation and land improvement on the activity of the soil microflora with reference to the effect of the latter on crop yield.

Great problems confront microbiologists in the task of increasing livestock production. They must continue to cooperate closely with other specialists in studies aimed at improving ensilage methods and providing collective and state farms with scientifically based recommendations which will ensure a reduction of the losses in ensilage and an improvement in the quality of the silage. Serious attention must be given to the devising of measures for enriching fodders in proteins, vitamins, antibiotics and other valuable substances, and to the practical adoption of such measures in animal husbandry.

There are no doubts as to the great effectiveness of a number of antibiotics in animal husbandry, particularly in the feeding of young stock.

A most important task is the improvement in the technology of manufacture of approved fodder antibiotics so that the stock-raising industry will be provided with highly effective, standard preparations. The mode of action of antibiotics will have to be thoroughly studied.

Research aimed at finding antibiotics and other biologically active substances suitable for use in agriculture is assuming great importance. Microbiologists must devote considerable attention to the devising of scientifically based measures for the prevention of waste in agriculture. There must be a more intensive adoption in agricultural practice of antibiotics and microbial preparations which have proved to be effective in the control of serious diseases of crops. Of no less importance is the wide practical adoption of microbial and viral preparations for the control of insect pests of crops and forests.

Crucial problems confront veterinary microbiologists in the matter of the further improvement of exist-

ing measures of combating infectious diseases of farm animals and in the development of new and more effective measures.

Closer liaison with production and vigorous, insistent efforts to secure the most rapid practical adoption of the results of scientific research still constitute one of the most important tasks of our scientists.

Thorough study, generalization and popularization of the experience and achievements of innovators and leading agriculturists are of considerable value.

The staffs of agricultural colleges are faced with the highly responsible task of reorganizing advanced education on the basis of closer liaison with the life and practice of the country's agriculture. It must be remembered that the training of fully qualified specialists requires that there must be a union of theory and practice in the instruction of the students. The unity of theory and prac-

tice, the experience of leading agriculturists and the achievements of advanced science must constitute the basis of the training of fully qualified specialists.

Guided by the decisions of the January Plenum, our microbiologists must check over and supplement their research plans and link them more closely with the urgent problems of agriculture. There is no doubt that Soviet microbiologists will direct all their efforts, knowledge and creative energy toward the speediest fulfillment of the resolutions of the Plenum and will make their contribution to the task of providing the country with an abundance of agricultural products.

Let us meet the 22nd Congress of the Communist Party of the Soviet Union with new advances in the development of Soviet microbiology and with decisive achievements in the development of all branches of agriculture!

EFFECT OF X RAYS ON PHOSPHORUS AND NITROGEN METABOLISM OF *Azotobacter agile* strain 22-D

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It is known that ionizing radiation has a complex biological effect on microorganisms and alters their normal metabolism (Meisel' [1955, 1958]). Phosphorus and nucleic acid metabolism, particularly the latter, are among the most sensitive to radiation (Pomoshchnikova [1955, 1956]; Katchman and Spoerl [1955]; Katchman, Fetty and Busch [1959]; Spoerl and Looney [1958]; Spoerl, Looney and Kaznierczak [1959]).

Recent information indicates that ionizing radiation, particularly ultraviolet rays, affects the synthesis, physicochemical structure, heterogeneity, and biological properties of nucleic acids, mainly DNA (Suzuki and Ono [1959]; Kameyama and Suzuki [1960]; Stuy [1958, 1959]).

However, there is practically no information on the change in the nucleotide composition of RNA and DNA in microorganisms exposed to ionizing radiation, and to X rays in particular.

In the present work we investigated the effect of various doses of X rays on the nitrogen and phosphorus metabolism of *Azotobacter agile* strain 22-D. We were particularly interested in discovering the effect of radiation on the nucleotide composition of RNA and DNA in *Azotobacter*.

MATERIAL AND METHODS

For this work we used *A. agile* strain 22-D (from the museum of the Microbiology Department of Moscow State University). The cultures were grown in a modified liquid Burk medium (Belozerskii, Zaitseva, et al. [1957]) for two days at 30°C on shakers. The live cells were separated from the nutrient, thoroughly washed with sterile water, and irradiated in a dense aqueous suspension. The cells were irradiated by a therapeutic X ray tube with no filters, at voltage 180 kv, current 15 ma, and dose rate 2100 kr/min. Doses of 5, 25, and 40 kr were used.

The cultures for the chemical analyses were not taken immediately after exposure, but after further cultivation for 24 hr in a fresh medium, when the changes in the chemical composition of the cells had become more pronounced. For comparison with the irradiated cells we analyzed a control culture grown for the same time. Radioactive orthophosphate (0.5 mC of $K_2H^{32}P_4O_{10}$

per liter) was added to the medium an hour before the samples were taken.

Fractionation of the acid-soluble and acid-insoluble phosphorus compounds and determinations of the specific activities of their phosphorus were carried out by the methods described in previous papers (Zaitseva, Belozerskii and Novozhilova [1959, 1960]). Mononucleotides were determined in an extract with trichloroacetic acid from the decrease of phosphorus, after their absorption on "Carboraffin" carbon. Soluble polyphosphates were determined after precipitation with Ba^{++} ions at $pH=4.5$, and phosphoric esters at $pH=8.2$ with the addition of four volumes of alcohol.

The phospholipid fraction was prepared by repeated extraction of the bacterial mass at room temperature with an alcohol-ether (3:1) mixture.

Acid-soluble phosphorus compounds were separated into RNA and DNA fractions by a slightly modified Schmidt and Thannhauser method (Zaitseva and Belozerskii [1957]). RNA nucleotides were separated by paper electrophoresis (Vanyushin and Belozerskii [1959]) and ion-exchange chromatography on Dowex-I (X 10) (Zaitseva, Belozerskii and Novozhilova [1960]). We used the method of gradient elution of ribonucleotides with 1 N and 4 N formic acid (Hurlbert, Schmitz, Brumm and Potter [1954]). In the calculation of the quantity of RNA mononucleotides separated on the columns we used the following millimolar extinction coefficients at $pH=2.0$: 13.8 for adenylic acid, 12.6 for guanylic, 9.9 for uridylic, and 6.8 for cytidylic.

To determine the specific activity of RNA nucleotides we selected fractions with the greatest optical density at $\lambda=260 m\mu$. After combustion of the mononucleotide with concentrated sulfuric acid, the quantity of orthophosphate and its specific activity were measured by the usual method (Zaitseva, Belozerskii and Novozhilova [1960]).

The quantity of DNA was determined by spectrophotometer (Spirin [1958]), and also by Dische's chemical method as modified by Burton [1956]. The radioactivity of the phosphorus of the total DNA was measured in preparations of its Na salts. The nucleotide

composition of DNA (Na salt) was investigated by paper chromatography (Kirby [1955]).

Phosphorus was determined by Berenblum and Chain's method as modified by Weil-Malherbe and Green [1951], nitrogen by the micro-Kjeldahl method, amino groups of free amino acids by Yemm and Cocking's method [1955], protein by the biuret reaction (Gornall, Bardawill and David [1949]), polysaccharides with anthrone from glucose (Zaitseva and Afanas'eva [1957]), total lipids by the weighing method after concentration of an alcohol-ether extract. Since the change in the size of cells of *A. agile* under the action of X rays was extremely irregular, all the analytical calculations were made per mg of dry weight of bacteria, and not per cell. The number of cells was determined by Winogradskii's counting method.

RESULTS

Figure 1 shows that exposure to a dose of 5 kr has no effect on the development of *A. agile*, whereas a dose of 10 kr suppresses cell division by approximately 50%. There is an almost complete cessation of multiplication for 30 hr after exposure to doses of 25 or 30 kr, and for 40 hr after exposure to 40 kr. The change in the size of irradiated cells of *A. agile* is irregular, but the majority of them are slightly larger than the controls. In this respect *Azotobacter* differs from certain other microorganisms.

Zaitseva, Agatova and Belozerskii [1961] showed earlier that in *Azotobacter vinelandii* oxidative phosphorylation was the process most sensitive to X rays, but the least sensitive processes were nitrogen fixation and respiration, particularly the latter. A clear illustration of the derangement of oxidative phosphorylation in *A. agile* due to X ray doses of 25 kr and 40 kr is the reduction in the quantity of labile phosphorus in the nucleo-

tides, which are represented in *Azotobacter* mainly by the ATP-ADP system (Table 1).

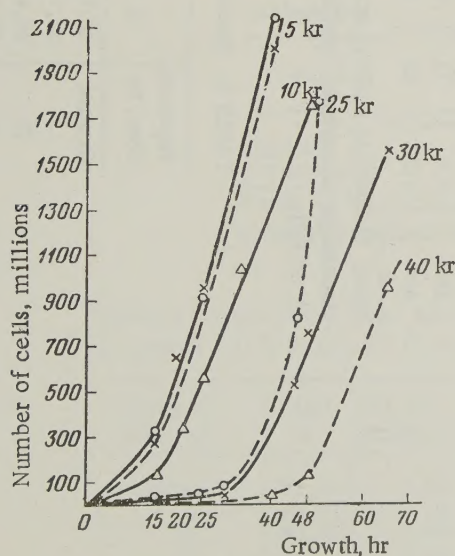
A dose of 5 kr not only did not inhibit but even caused some stimulation of esterification of orthophosphate. The derangement of the important energy-supplying process — oxidative phosphorylation — in *A. agile* affected the phosphorus metabolism as a whole. There were particularly pronounced changes in the quantity of orthophosphate, the content of which fell to 60% of that of the control in *Azotobacter* exposed to 25 kr, and to 27% in *Azotobacter* exposed to 40 kr. This fact can be attributed to slower assimilation of external orthophosphate from the medium or, on the other hand, to increased washing out of intracellular orthophosphate. Possibly these two processes occur together in irradiated *Azotobacter* cells and lead to a reduction in the quantity of total organic phosphorus compounds [see total phosphorus (P) in Table 1].

The inhibition of the synthesis of complex organic compounds by *A. agile* under the action of X rays was greatest in the case of nucleic acids and of DNA in particular. For instance, a dose of 5 kr did not reduce the quantity of RNA, whereas the quantity of DNA in this case was reduced to 80% of that of the control. Exposure of *Azotobacter* cells to doses of 25 or 40 kr reduced the synthesis of RNA by 25-30%, and that of DNA by 60%. The derangement of nucleic acid synthesis in irradiated cells of *A. agile* was also indicated by the pronounced reduction in the rate of renewal of R-nucleic acids and of DNA in particular, the incorporation of phosphorus in which practically ceased when the cells were exposed to a dose of 25 kr (Table 1).

A study of the rate of renewal of phosphorus in individual nucleotides of RNA (Table 2) showed that exposure to 5 kr did not cause any changes of specific activity in comparison with the control. An increase of exposure dose to 25 kr and, especially, to 40 kr resulted in a considerable reduction in the rate of renewal of phosphorus in all the RNA nucleotides, the specific activity of R-uridylic acid being most reduced. This would appear to indicate some derangement of normal RNA metabolism and, perhaps, mainly of those metabolic links involved in the biosynthesis of uridylic nucleotides.

However, the considerable alteration of nucleic acid metabolism in *A. agile* under the action of X rays had no effect on the qualitative and quantitative nucleotide composition of the total RNA (Table 3) and total DNA (Table 4).

Thus, we can infer from the given data that the disturbances in nucleic acid metabolism which are brought about by the action of X rays are connected not so much with changes in the nucleotide composition, which remains constant, as with the quantitative aspect of nucleic acid synthesis. The reduction of the nucleic acid content at exposure doses 25 and 40 kr was accompanied by an increase in the quantity of acid-soluble nucleotides (Table 1). This can be attributed, on one hand, to



Growth of *A. agile* before and after exposure to X rays.

TABLE 1. Quantity and Specific Activity of Phosphorus in Various Compounds in Control and X-Irradiated Cultures of *A. agile*

Exposure dose, kr	Acid-soluble compounds						Acid-insoluble compounds					
	Orthophosphate		P of soluble polyphosphates		Nucleotides		P of sugar esters		P of RNA		P of DNA	
					P _{tot}	P _{lab}					P of insoluble polyphosphates	
	1	2	1	2	1	2	1	2	1	2	1	2
Control	2.23	35.3	0.58	12.0	1.18	18.0	0.34	30.0	1.30	15.0	1.80	36.0
5	2.15	38.0	0.92	18.0	1.20	20.0	0.37	33.0	1.18	17.0	1.62	35.0
25	1.31	30.1	1.15	23.0	1.32	17.0	0.24	25.0	0.85	14.0	1.50	24.0
40	0.60	26.5	1.76	21.0	1.50	15.0	0.18	23.0	0.67	12.0	0.82	22.0
									3.74	7.7	0.88	0.6
									5.25	11.3	1.52	3.4
									5.60	11.8	1.29	2.1
									3.97	8.0	0.96	0.8
									3.74	7.7	0.88	0.6
											1.90	3.0
											2.30	4.2
											1.98	4.0
											1.93	3.5
											1.90	3.0
											21.27	13.0
											20.57	14.0
											17.22	10.0
											15.09	7.0

Note: 1) Quantity of phosphorus in $\mu\text{g}/\text{mg}$ of dry weight of bacteria; 2) specific activity of phosphorus in counts/min/ μg P.

TABLE 2. Specific and Relative Specific Activity of P^{32} in Nucleotides of RNA in *A. agile*

Exposure dose, kr	C		A		G		U		RNA	
	1	2	1	2	1	2	1	2	1	2
Control	11.3	100	14.8	100	11.0	100	10.4	100	11.4	100
5	11.8	104	15.0	101	11.2	102	10.0	96	12.0	105
25	8.0	71	11.5	78	8.1	73	7.1	68	8.7	76
40	7.7	68	11.1	75	7.8	71	5.4	52	8.0	70

Note: 1) Specific activity in counts/min/ μg P of nucleotide; 2) relative specific activity as percentage of control. Symbols in Tables 2 and 3: C) cytidylic acid; A) adenylic; G) guanylic; U) uridylic.

TABLE 3. Nucleotide Composition of RNA of *A. agile* (mol.-% of nucleotides)

Exposure dose, kr	C	A	G	U	Pu**/Py**	G+U A+U	G+C A+U
Control	25.7	24.4	30.4	19.5	1.21	1.00	1.28
5	25.7	24.3	30.6	19.4	1.22	1.00	1.29
25	26.4	24.5	30.0	19.1	1.20	0.96	1.29
40	25.6	24.6	29.8	20.0	1.20	0.99	1.24

* Pu) purine nucleotides; ** Py) pyrimidine nucleotides.

the inhibited synthesis of high-molecular polynucleotides and, on the other, to their degradation to low-molecular fragments.

Since oxidative phosphorylation in *Azotobacter* is greatly disturbed by radiation, we can infer that there will be no synthesis of polyphosphates in these conditions either. In fact, after exposure to X ray doses of 25 kr or 40 kr, particularly the latter, there was a reduction in the quantity of acid-insoluble polyphosphates and a correspondingly large increase in soluble polyphosphates (Table 1). The total quantity of insoluble and soluble polyphosphates in irradiated cells of *A. agile* was approximately equal to their sum in the control cells, which might indicate depolymerization of high-molecular polyphosphates to low-molecular compounds. The actual occurrence of this process in irradiated *Azotobacter* cells was indicated by the reduction of the specific activity of the phosphorus of insoluble polyphosphates. This was accompanied by a parallel increase in the specific activity of soluble polyphosphates. This degradation of polyphosphates did not lead to orthophosphate, but to larger molecules, since the quantity and specific activity of orthophosphate in the irradiated cells did not increase but, on the contrary, were greatly reduced (Table 1).

Exposure to X rays caused a reduction in the protein content, whereas the free amino acid fraction was very considerably increased (Table 5).

The increase of free amino acids became appreciable even at the low dose 5 kr, although the quantity of protein in this case was practically unchanged. This may be explained by the slower utilization of amino acids for synthesis of the latter. The 20-22% reduction of protein content below that of the control at doses 25 and 40 kr was probably due, not only to the inhibition of synthesis, but also to the depolymerization of the protein

molecule with the result that the quantity of soluble nitrogenous substances of the amino acid and, possibly, peptide type was increased by a factor 2-2.5.

Thus, the effect of X irradiation of *Azotobacter* cells shows a general pattern, consisting in the reduction of the quantity of high-molecular compounds and a corresponding increase in low-molecular products. X rays also had a considerable effect on the carbohydrate and lipid metabolism of *A. agile*.

Table 1 shows that the quantity of phospholipids and acid-soluble phosphoric esters of sugars, particularly the latter, was considerably reduced in irradiated cells. On the other hand, irradiation led to an increase in intracellular polysaccharides and lipids, the quantity of which in *Azotobacter* exposed to 40 kr was almost one and a half times greater than in the control (Table 5).

Thus, the derangement of the synthesis of biologically important substances — proteins and nucleic acids — in irradiated *Azotobacter* cells is compensated by an increased production of polysaccharides and lipids.

The authors express their deep gratitude to Prof. A. N. Belozerskii for his interest in this work.

SUMMARY

Exposure to X rays has a considerable effect on the nitrogen, phosphorus and, most of all, nucleic acid metabolism of *Azotobacter agile* strain 22-D.

The derangement of oxidative phosphorylation due to X rays leads to a change in the normal synthesis of protein, RNA and, in particular, DNA.

However, the nucleotide composition of RNA or DNA in irradiated cells of *A. agile* does not differ from that of the control.

Exposure to X rays inhibits the biosynthesis and reduces the degree of polymerization of complex nitrogen

TABLE 4. Nucleotide Composition of DNA of *A. agile* (mol. - % of bases)

Culture	G	A	C	T	Pu/Py	$\frac{G+T}{A+C}$	$\frac{G+C}{A+T}$
Control	27.5	22.2	26.4	23.8	0.99	1.05	1.17
Exposed to 40 kr	27.8	22.2	26.0	24.0	1.00	1.07	1.16

Note: G) Guanine; A) adenine; C) cytosine; T) thymine; Pu) purine bases; Py) pyrimidine bases.

TABLE 5. Quantity of Total Amino Nitrogen of Free Amino Acids and Basic Substances in Control and X-Irradiated Cultures of *A. agile* (in μg per mg of dry weight of bacteria)

Exposure dose, kr	Total N	NH ₂ of amino acids	Protein	RNA	DNA	Polysaccharides	Lipids
Control	81.50	1.01	407.80	54.75	15.19	118.35	117.00
5	82.50	1.48	397.30	58.40	12.88	150.48	131.21
25	80.00	1.98	328.20	41.41	9.55	189.36	145.45
40	—	2.62	317.80	38.92	8.80	206.40	152.28

and phosphorus compounds. This leads to a reduction in the quantity of protein, nucleic acids and acid-insoluble polyphosphates and to an increase in free amino acids, mononucleotides and acid-soluble polyphosphates. The synthesis of polysaccharides and lipids in irradiated cells of A. agile does not cease, but is even enhanced to some extent.

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COLONY STRUCTURE IN A HIGHLY ACTIVE RADIATION MUTANT OF *Actinomyces streptomycini* Kras.

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Spontaneous and induced hereditary changes in actinomycetes are accompanied by pronounced changes in the morphological structure of the colony.

A cytological analysis of transverse sections of colonies of the natural strain 58 and a similar strain LS-3 of *Actinomyces streptomycini* Kras. showed that the super-substrate part of colonies of these strains was characterized by a layered arrangement due to the shortness of the complete life cycle of one generation of mycelium, during which it does not manage to consume the nutrient substances in the region of the colonies. These nutrient substances are used by several generations of mycelium, each of which consists of a regular alternation of two types of hyphae — prostrate (vegetative feeding hyphae) and vertically growing hyphae of the aerial mycelium (vegetative and spore-forming hyphae).

As investigations showed, a very important condition for abundant sporulation by two or three generations of the aerial mycelium typical of strains 58 and LS-3 was the intense reproduction of DNA-rich nuclear elements in the hyphae of the second phase of development (Prokof'eva-Bel'govskaya and Shamina [1960]).

The aim of the present investigation was to discover the cytological structure of colonies of strain LS-1, a highly active radiation mutant of *A. streptomycini*, and to compare the special features of its structure with those of the natural strain 58 and a similar strain LS-3.

MATERIAL AND METHODS

The study was made with a culture of *Actinomyces streptomycini* Kras., strain LS-1, obtained from the Museum of Strains of the All-Union Antibiotics Research Institute (VNIIA).

Strain LS-1 was obtained in the selection laboratory of VNIIA by a stepwise selection procedure involving exposure to ultraviolet and X rays at different stages. Its antibiotic activity lay between 2000 and 2500 units/ml; it was a typical radiation mutant with a high capacity for streptomycin production.

The material for the investigation consisted of colonies 2-25 days old on pea agar, the medium normally employed for cultivation of this strain in the selection laboratory of VNIIA.

Colonies cut out from the agar medium were fixed in Carnoy's fluid or by the Navashin technique in the usual way, after which they were brought through alcohols and xylene and embedded in paraffin. Microtome sections (2-3 μ thick) of the colonies were stained with Heidenhain's iron hematoxylin, gentian violet by the Feulgen method, and with methyl green and pyronine. The methods were described more fully in a previous paper (Prokof'eva-Bel'govskaya and Shamina [1960]).

RESULTS

On agar medium the colonies of strain LS-1 are deformed, distinctly convex, plicate, weakly sporulating, and white in color.

In transverse section two-day-old colonies are similar to colonies of strains 58 and LS-3 of the same age (Prokof'eva-Bel'govskaya and Shamina [1960]) as regards general structure and the cytological features of the hyphae. The colonies consist of substrate mycelium with loosely arranged hyphae and a supersubstrate part in which the hyphae are densely interwoven (Fig. 1, 1).

The hyphae of the substrate mycelium are weakly branched, and the protoplasm is weakly basophilic. The rod-like nucleoids stain weakly by the Feulgen procedure. The hyphae of the substrate mycelium at this period penetrate only a certain depth into the medium and do not grow below this limit. Just as in the case of strains LS-3 and 58, the hyphae in the lower part of the substrate mycelium grow horizontally and form a distinct boundary with the medium.

In strain LS-1, as distinct from strains 58 and LS-3, partial autolysis of the hyphae of the primary mycelium begins on the second day of growth. This occurs both in the substrate and supersubstrate parts of the colony. The autolysis rapidly spreads throughout the whole mass of the colony. In the region enriched by the autolysis products the young basophilic hyphae of the secondary mycelium begin to grow (Fig. 1, 2). Passing into phase II, these hyphae develop particularly thickly in the lower region of the substrate part of the colony (Fig. 1, 3). Nuclear elements are clearly visible in the weakly basophilic cytoplasm of these thickened hyphae.



Fig. 1. *Actinomyces streptomycini*, strain LS-1; transverse section of colony ($2\ \mu$); 1, 2, 3) two days of growth; 4, 5) six days of growth. 1) General structure of colony; 2) early autolysis and growth of secondary hyphae in substrate part of colony; 3) thickened hyphae of phase II of development in substrate mycelium, large nuclear elements visible; 4) general structure of colony — thickly developed supersubstrate vegetative mycelium formed by prostrate hyphae below (Ip), by network of hyphae above (ne), and by bunches of vertically growing hyphae on top (vb); 5) bunches of vertically growing vegetative hyphae in upper part of colony. Methyl green and pyronine.

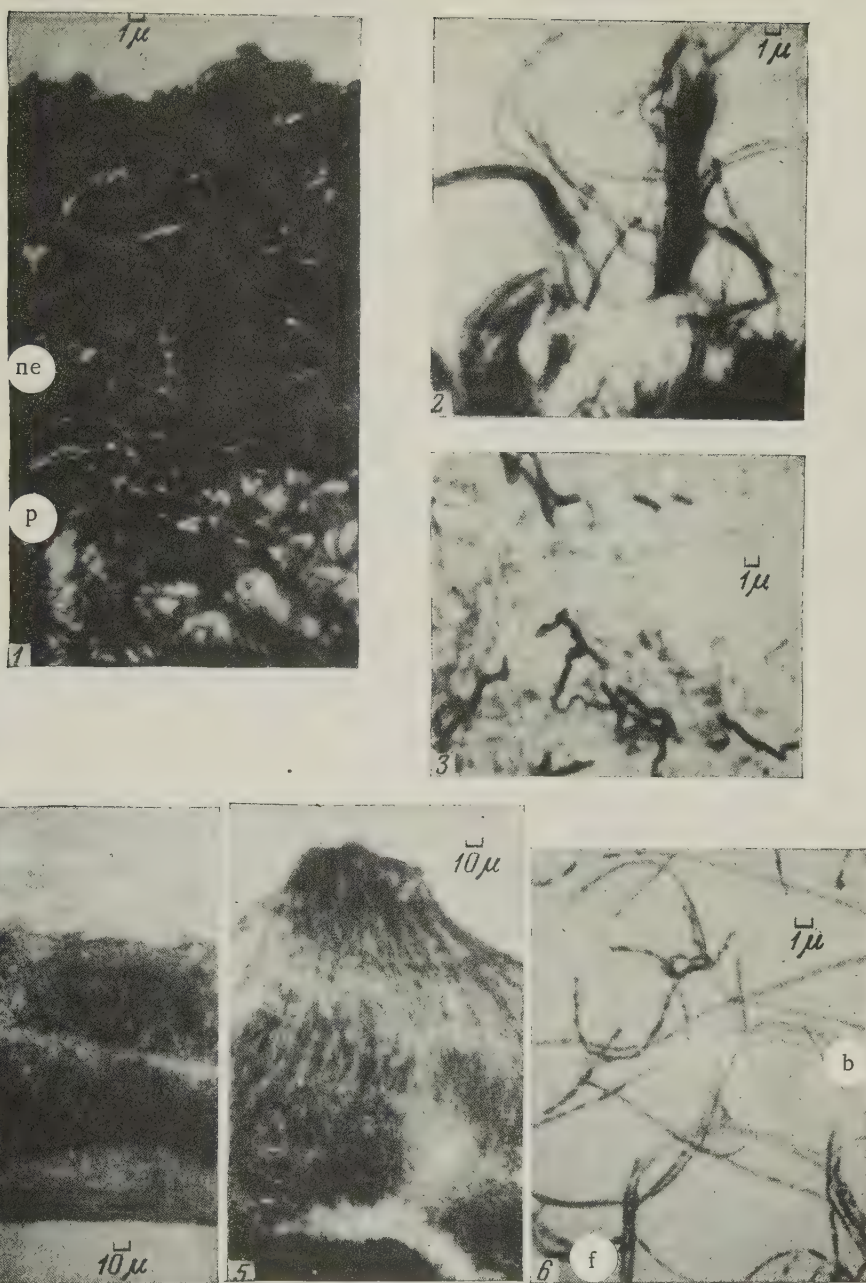


Fig. 2. *Actinomyces streptomycini*, strain LS-1; transverse section of colony (2μ); 1, 2) 10 days of growth; 3, 4, 5, 6) 25 days of growth. 1) Smooth surface of colony formed by thick layer of prostrate vegetative hyphae (p), ne – network of hyphae; 2) bunches of vertically growing vegetative hyphae piercing surface of colony in places; 3) thickened secondary hyphae in central zone of autolyzed substrate mycelium; 4) general structure of colony – two generations of mycelium formed by prostrate (p) and vertical (v) hyphae; bunches of vegetative hyphae (b) on surface of colony; 5) bunches of vertically growing hyphae forming elevation on colony surface; 6) print of surface mycelium of colony – fine hyphae of surface bunch (b), fragments of thickened secondary vegetative hyphae (f). Methyl green and pyronine.

In the region next to the agar the supersubstrate mycelium of the two-day-old colony consists of a layer of horizontally growing hyphae. Above this there is a network of interwoven hyphae and, finally, the surface of the colony is formed by free hyphae which show a tendency to grow upward (Fig. 1, 1). These hyphae contain nuclear elements which stain deeply by the Feulgen procedure.

On the sixth day of growth the substrate part of the colony has the same limited region of growth as before. This is obviously due to the accumulation here of products of the activity of the colony.

The supersubstrate part of the six-day-old colony is formed of several layers of mycelium. At the agar surface there is a layer of horizontally growing hyphae pressed close to the substrate. Above this there is a network of mycelium alternating with indistinct horizontal layers of hyphae. The ascending hyphae grow in bunches and form the smoothly plicate surface of the colony (Fig. 1, 4, 5). There is no free vertical growth of aerial hyphae on the surface of the colony and no sporulation. The formation of such a surface is brought about by the preferential growth of the hyphae in the region richest in easily assimilable nutrient substances — such regions are the surface zone of the colony and the zone of the bunches of hyphae.

Thus, by the sixth day the nature of the growth and structure of the colony of strain LS-1 differs distinctly from that in strains LS-3 and 58, where vertical, freely growing sporophores develop on the surface of the colony at this time. These differences are maintained throughout the subsequent development of the culture.

On the tenth day the substrate mycelium consists of a system of autolyzed hyphae of the primary mycelium and thick basophilic hyphae of the secondary mycelium, which grow freely into the medium. The nuclear elements in the latter do not give a nucleal reaction, and their DNA content is greatly reduced. This is a characteristic feature of the mycelium of actinomycetes in the period of rapid growth of the cytoplasmic body of the cell (Prokof'eva-Bel'govskaya, Pestereva and Rudaya [1956]; Prokof'eva-Bel'govskaya and Popova [1959]). The supersubstrate part of the colony consists of an intricate alternation of layers of thick, highly basophilic, prostrate hyphae and a network of mycelium with slightly ascending hyphae (Fig. 2, 1). Large bunches of vertically growing hyphae penetrate to the colony surface, which is formed by prostrate hyphae. These bunches form small elevations and folds on the surface of the colony. In some parts large brush-like bunches pierce through the dense layer of horizontal growth on the surface of the colony (Fig. 2, 2). The cytoplasm of these hyphae is basophilic, and the nuclear elements stain weakly by the Feulgen method. The growth of the hyphae in a bunch probably leads to an increased concentration of enzymes and, hence, of more easily assimilable substances in the microzone of the bunch; herein resides the adaptive na-

ture and advantage of this type of growth over that of single free hyphae.

By the 25th-32nd day of growth the secondary mycelium developing on the products of autolysis of the primary hyphae in the substrate part of the colony proliferates strongly within the central region of the substrate part of the colony (Fig. 2, 3).

In the supersubstrate part of the colony gaps appear owing to partial autolysis in the layers of prostrate hyphae, and consequently the sections of the colony clearly reveal the layered structure due to the alternation of horizontal and network growth, just like that observed in strains LS-3 and 58 (Fig. 2, 4). Distorted short hyphae, which break up into fragments of different thickness, develop on the autolysis products in the upper layers of the colony. These fragments contain basophilic cytoplasm and poorly developed nuclear elements with a low DNA content (weak nucleal reaction). These fragments are clearly seen in sections and on prints of the colonies (Fig. 2, 6). The small mounds which had appeared on the surface of the colony by the tenth day and which were formed by bunches of vertically growing hyphae produce large elevations by the 25th-32nd day (Fig. 2, 5). A weakly developed aerial mycelium in the form of a slight fluff appears in different parts of the colonies by the 25th day. This mycelium consists of very fine, loosely growing hyphae containing small granules of volutin. The sparsely scattered small nuclear elements in them hardly stain at all by the Feulgen method (Fig. 2, 4).

In the multiplication of LS-1 by means of the aerial mycelium the main role is presumably played by the fragments of hyphae of the secondary mycelium, which contain nuclear elements giving a positive nucleal reaction.

Thus, a cytological study of the colony structure showed that a complete cycle of development is a characteristic feature of the natural strain 58 and strain LS-3. The DNA-rich nuclear elements in the hyphae of the aerial mycelium are rapidly reduplicated, thus stimulating complete sporulation in two or three generations of aerial mycelium (Prokof'eva-Bel'govskaya and Shamina [1960]). The radiation mutant — strain LS-1 — is characterized by a pronounced alteration of the life cycle typical of natural cultures of this species. The development of the colony of strain LS-1 takes place in two stages. The colony is at first enriched with DNA and RNA during the development of the primary mycelium. After the early autolysis of the latter the colony uses the products of its previous activity for the rapid and prolonged vegetative growth of the secondary mycelium in the substrate and supersubstrate parts of the colony. The supersubstrate mycelium is rich in RNA and contains indistinct nuclear elements with a low DNA content. Such mycelium remains for a long time in the vegetative phase. It has a high capacity for growth and produces two or three generations of prostrate hyphae, network

hyphae, and vertically growing bunches of hyphae. However, the inhibited reproduction of nuclear elements during the vigorous growth of the cytoplasmic body of the cell and the low DNA content of the nuclear elements does not allow intense sporulation. When grown in submerged culture conditions, which ensure the biosynthesis of streptomycin, strain LS-1 possesses the same cytological features of growth and development (Prokof'eva-Bel'govskaya, Alikhanyan, Kapitonova and Erokhina [1957]). It is quite probable that the high capacity of strain LS-1 for streptomycin biosynthesis is due to its inherited ability for prolonged vegetative growth. We cannot infer from this, however, that any strain with a prolonged vegetative phase will be a very active producer of antibiotic.

SUMMARY

The development and structure of colonies of a highly active radiation mutant *Actinomyces streptomycini* Kras., strain LS-1, were studied from 2-3 μ sections. Distinct differences were discovered in the microscopic morphology of colonies of this strain and the intensely sporulating colonies of the natural strain 58 and a similar strain LS-3 grown on agar medium. These differences are due to the great prolongation of the period of growth of the highly basophilic vegetative hyphae of the

prostrate, network and vertical growth in the supersubstrate part of the colony. The bunches of vertically growing hyphae do not pass into the stage of free aerial growth, despite their tendency to penetrate through the layer of prostrate hyphae to the surface of the colony. The bunches of hyphae form folds and elevations on the surface of the colony. These hyphae contain a few minute nuclear elements with a low DNA content, and hence sporulation is suppressed. Thus, the emergence of the LS-1 mutant is presumably due to hereditary changes in RNA and DNA metabolism and to the consequent change in the duration of the phases of the life cycle of the actinomycete. The changes in metabolism lead to changes in the microscopic morphology of the colonies.

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ACQUISITION OF RADIORESISTANCE BY MICROBE CELLS INHABITING MEDIA WITH INCREASED LEVELS OF NATURAL RADIATION

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A number of authors have proved that, by use of sublethal doses, it is possible to raise artificially the radioresistance of an organism to lethal doses of ionizing radiation (Betz, 1950; Cronkite 1950; Kiselev, Buzini, Nikitina, 1956; Graevskaya, Keilina, 1956; and others).

It was established that the radioresistance thus acquired is of a nonspecific nature, and can be caused by factors other than the action of radiation (Klemparskaya, 1957). This radioresistance invoked by preliminary irradiation is concomitantly accompanied by a lowered sensitivity to other harmful agents (Kiselev, Semina, 1959).

An analysis of this radioresistance has shown that its advent is, to some extent, connected with a change in the general immunological defense mechanisms of the organism, and specifically with the formation of antibodies against the normal body microflora and the organism's own denatured tissue protein and with changes in the basic intracellular connective tissue matter (Kiselev, Semina, 1959).

Whether this radioresistance is connected with a changed sensitivity to radiation in the somatic cells themselves remains an open question, subject to specialized study. Besides, because of the action of ionizing radiation, some researchers altogether deny the possibility of adaptation by organisms to it (Lebedinski, 1957). Further experimental studies on the question of adaptation by organisms to radiation, if they are to be of any significance, must be conducted on the cellular level, because this is the only way we can reach a theoretical and general biological solution of this problem. With this point in mind, microorganisms present a convenient subject for such investigation. But we have scant and insufficiently convincing data concerning radioresistance in microbial mutants which are produced by heavy doses of ultraviolet or roentgen rays (Witkin, 1947; Maisin et al, 1956; Pershina, 1957).

Our research was conducted in two directions. On one hand it dealt with the possibility of experimental

adaptation of an intestinal alkalizer, *Alcaligenes faecalis* (*Bacterium alcaligenes*), to a chronic and an acute action of ionizing radiation; on the other hand, we investigated the development of radioresistance in microbes resulting from their prolonged natural habitation in radioactive media, i.e., the influence of increased levels of natural radiation.

A primary aim of this work was to determine the effects of prolonged habitation in water sources with increased radiation levels on the radioresistance, viability, and certain enzyme systems of the microbial cell.

MATERIAL AND METHODS

In the summer of 1957 we isolated various microorganisms from radioactive and nonradioactive Caucasian mineral water sources (Piatigorsk, Zheleznovodsk). The radioactivity of the waters from which the microbes were isolated was due to the presence of radium, and varied, according to the source, between 30 to 362 $\mu\text{r/hr}$. The water sources could be classed into three categories, according to their radioactivity: the radioactive waters (362-120 $\mu\text{r/hr}$), the slightly radioactive (30-40 $\mu\text{r/hr}$), and the nonradioactive. Radioactivity determinations were made with the ST-42 radiometer.

All the isolated microorganisms were aerobic cocci or bacilli, and they grew on meat-peptone agar. We investigated 150 cultures. After a detailed morphological, cultural, and biochemical study, each of the microorganisms was determined down to its species according to Krasil'nikov's classification (1949).

The identification procedures established that most of the microorganisms belonged to the genera *Micrococcus* (*M. candidus*, *M. citreus*, *M. cinnobarinus*) and *Bacillus* (*B. intermedius*, *B. adhaerens*, and others). Of the identified cultures isolated from radioactive and nonradioactive sources, we selected eleven pairs of analogs from radioactive, weakly radioactive, and nonradioactive waters. Of this number of 11 paired strains, 6 were cocci and 5 were rods. These cultures were select-

ed for a comparative study of their radioresistance and of their relationship to radiomimetic substances (em-bichin, H_2O_2). At the same time, we studied the activity of certain enzymes (oxidase, peroxidase, dehydrase) in these microorganisms.

The radioresistance of the isolated microorganisms was determined by the size of the radiation dose corresponding to LD_{50} . The microorganisms were irradiated with the roentgen apparatus RUM-7, using 50 kv, 5 ma, no filter or tube, at a distance of 9 cm. The strength of the emission dose was 7,700 r/min. The cultures were prepared for irradiation as follows. The test organisms, grown on agar for 18 hr, were washed off with a physiological solution, shaken for 10 min, washed once again to eliminate traces of the medium, and diluted to a concentration of 500 million cells per ml. Equal volumes of microbial suspension were poured into special standard sterile vessels and covered with sterile cellophane. To avoid variations in depth of the irradiated medium, the vessels were placed in an exactly horizontal position during the irradiation.

Each of the tested cultures was subjected to the action of roentgen rays in the entire emission range of 7,700 to 30,800 r. Survival rate of the irradiated cells was determined by a colony count on inoculations made in 3-4 different dilutions of the microbial suspension after its subjection to its respective radiation dose. Each experiment was repeated twice.

Simultaneously, a study of the sensitivity of the cultures to hydrogen peroxide and to neoembichin was conducted by the serial dilution method in fluid medium. The concentration of hydrogen peroxide varied within the range 0.0001 - 0.5%. The concentration of neoembichin varied from 100 to 2000 $\mu\text{g/ml}$.

The comparative study of enzyme activity concerned those enzyme systems related to the intracellular oxidation-reduction processes. Enzyme activity was determined in a suspension of quiescent microbial cells. Dehydrase activity was determined by Tunberg's method. As substrates for dehydrase activity we used mono- and disaccharides, multiatomic alcohols, organic acids. The substrates served as the control. Dehydrase activity was measured by the decoloration of methylene blue. Peroxidase activity determinations were conducted by Wilshteter's method, based on colorometric change of the purpurogaline concentration. Intensity of coloration was measured in the FEK-1 photocolormeter. Catalase was determined by the method of Bach and Oparin. Catalase activity in microorganisms was expressed by the amount of undecomposed H_2O_2 , as determined by titration with 0.1 N $KMnO_4$.

RESULTS

The study of radioresistance in microbial cultures isolated from radioactive, weakly radioactive, and non-radioactive waters revealed significant differences between them. Table 1 presents data on determinations

TABLE 1. Radioresistance of Microbial Cultures Isolated from Radioactive and Nonradioactive waters

Cultures	Radioactivity of water source (in micro-roentgens/hr)	LD_{50} (r)
<i>M. cinnobarinus</i>	362	10627
<i>M. sulfureus</i>	362	8471
<i>M. sulfureus</i>	350	10270
<i>M. candidus</i>	194	11555
<i>B. adhaerens</i>	120	11298
<i>B. intermedius</i>	120	22596
<i>B. intermedius</i>	40	16942
<i>B. adhaerens</i>	30	1028
<i>M. sulfureus</i>	30	4369
<i>M. candidus</i>	nonradioactive	2313
<i>M. cinnobarinus</i>	"	1799
<i>M. sulfureus</i>	"	3084

of the semilethal dose (LD_{50})* of the most typical analogs among the microorganisms tested. From these results, we see that the most radioresistant microorganisms proved to be the ones isolated from the radioactive waters, whereas analogs of the same microbes isolated from nonradioactive or weakly radioactive water sources had a low resistance. This difference was observed in both the micrococci and the bacilli. The radioresistance of the micrococci isolated from radioactive waters proved to be 4-5 times greater than that of their non-radioactive analogs. A comparison of radioresistance in *B. adhaerens* isolated from radioactive and weakly radioactive waters showed an even more pronounced difference. Radioresistance of this microbe taken from radioactive waters was 10 times greater than that of its analog cultured from weakly radioactive water ($LD_{50} = 11,289$ r as compared to $LD_{50} = 1,028$ r). Significantly less difference in radioresistance was observed upon comparing cultures of *B. intermedius* from radioactive and nonradioactive water ($LD_{50} = 22,568$ r; $LD_{50} = 16,942$ r).

The data obtained show that, of the cultures tested, not a single one inhabiting high natural radiation levels proved less radioresistant than its nonradioactive analog.

Thus, our experiments with microorganisms from radioactive water, conducted by determining the LD_{50} of their roentgen ray irradiation, show that these microorganisms possessed a significant radioresistance as compared to their analogs from nonradioactive waters. The radioresistance of cultures isolated from weakly radioactive waters approached that of the microorganisms from nonradioactive water sources.

In studying the radioresistance of all the above cultures, concomitantly with the LD_{50} determinations we

* LD_{50} - Lethal radiation dose causing mortality in 50% of the microbial cells.

made a detailed study of their survival rate upon roentgen irradiations in the entire range of 2,500 to 38,500 r. Since we cannot present the data obtained for all 22 cultures tested, we shall limit ourselves to an examination of some typical survival curves of irradiated cultures from waters of varying degree of radioactivity, and from nonradioactive waters.

Figure 1 presents survival curves for *M. candidus*, *M. cinnobarinus* and *B. adhaerens* (isolated from radioactive and nonradioactive waters) after irradiation with various doses of roentgen rays. Survival of microbial cells of these cultures is expressed in percentage, and represents the number of irradiated cells capable of multiplication as compared to the total number of cells subjected to roentgen ray action. Figure 1 shows that the survival ratio for microorganisms from radioactive waters is significantly higher than for their analogs from nonradioactive waters.

Survival of micrococci from radioactive water (194 $\mu\text{r}/\text{hour}$) was 44% with a dose of 30,800 r; its analog from nonradioactive water had a 14% survival. Similar differences can be seen in the other cultures. A significantly smaller difference in cell radioresistance was observed when comparing microbial cultures isolated from weakly radioactive and nonradioactive waters. Figure 2 presents survival data for four cultures of *M. sulfureus*, two of them isolated from weakly radioactive waters (1 and 2); the other two from nonradioactive waters (1a and 2a). The higher the natural radiation level in the habitat of the microorganism, the greater was the observed difference in radioresistance.

It was interesting to determine whether this cellular radioresistance acquired during habitation in a radioactive medium is specific for radiation only, or whether this resistance is manifested toward other factors too — e.g., hydrogen peroxide and embichin. A study of microbes from radioactive and nonradioactive waters es-

tablished that the resistance to hydrogen peroxide was 1.5-2 times greater in cultures inhabiting a relatively highly radioactive medium (362-194 $\mu\text{r}/\text{hr}$) than in cultures isolated from nonradioactive waters (Table 2).

The stability to embichin, however, proved to be the same in the radioresistant cultures as in the less radioresistant ones. This circumstance is important in two respects. On the one hand, it indicates that the radioresistance acquired by the microbial cell is, as contrasted to the radioresistance of a complex organism, of a

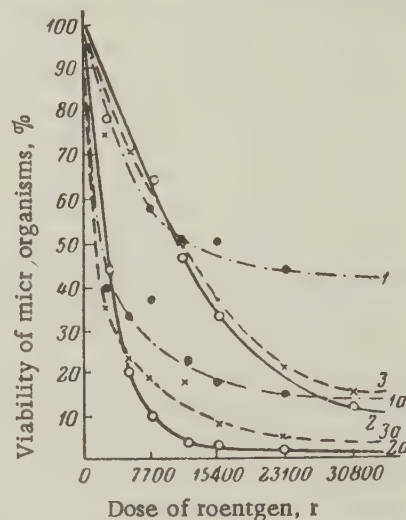


Fig. 1. Survival of microorganisms (from radioactive and nonradioactive water sources) upon irradiation with roentgen rays. 1) *M. candidus*; 2) *M. cinnobarinus*; 3) *B. adhaerens* isolated from radioactive water (activity 362, 194 and 120 $\mu\text{r}/\text{hr}$). Curves 1a, 2a, and 3a designate the same species of microbes inhabiting nonradioactive waters.

TABLE 2. Stability of Microbial Cultures Isolated from Radioactive and Nonradioactive Sources to Radiomimetic Substances, and the Peroxidase and Catalase Activity of these Cultures

Cultures	Radioactivity of the source ($\mu\text{r}/\text{hour}$)	Bactericidal dose of H_2O_2 (%)	Bactericidal dose of o-embichin ($\mu\text{g}/\text{ml}$)	Relative peroxidase content by the size of E (extinction)	Catalase number
<i>M. cinnobarinus</i>	362	0.1	400	1.2	6.5
<i>M. sulfureus</i>	362	0.08	200	1.3	4.9
<i>M. sulfureus</i>	350	0.06	300	1.2	0.9
<i>M. candidus</i>	194	0.14	1800	1.2	1.9
<i>B. adhaerens</i>	120	0.05	400	1.05	6.3
<i>B. intermedius</i>	120	0.03	1000	0.36	1.1
<i>B. intermedius</i>	40	0.02	300	0.83	4.9
<i>B. adhaerens</i>	30	0.03	250	0.92	0.68
<i>M. sulfureus</i>	30	0.08	500	1.1	4.7
<i>M. candidus</i>	non-radioactive	0.06	1400	1.2	0.5
<i>M. cinnobarinus</i>	"	0.05	300	1.1	6.5
<i>M. sulfureus</i>	"	0.05	500	1.4	5.9

more specific nature; on the other hand, it indicates the existence of very basic differences between the actions of embichin and radiation. The somewhat increased stability of radioresistant cultures to hydrogen peroxide is, apparently, connected with the fact that the formation of peroxides which occurs during the mechanism of radiation action on the cell has a certain significance.

The mechanism of radioresistance is an interesting question. One might suppose that the process of cellular adaptation to increased radiation levels, in either the natural habitation medium or in induced circumstances, would be accompanied by changes in the enzymes responsible for intracellular oxidation-reduction processes.

We conducted, therefore, a study of catalase, peroxidase, and dehydrase activities in the radioresistant and in the more radiosensitive analogs of the various microbial cultures (Table 2). The study of peroxidase and catalase gave no basis for concluding that the radioresistance is dependent on the activation of said enzymes. The dehydrase activity of radioresistant cultures likewise did not differ from the activity of their more radiosensitive analogs.

RESULTS

Ionizing radiation, depending on its dose, can either cause cell death, or a mutational process leading to variation of the microorganism. What direction this variation and further selection take in natural conditions is as yet not sufficiently clear. Likewise unclear is the evolutionary significance of these occurring transformations. Our experimental data have merely indicated that where increased levels of natural radiation exist, there probably

occurs a variation process in microorganisms leading to the occurrence of more radioresistant forms. In support of this hypothesis is the fact that microorganisms isolated from natural habitats with increased natural radiation levels (362-30 $\mu\text{r/hr}$) possess a greater radioresistance than their analogs from nonradioactive water.

Radioresistance in the various microorganisms was increased 3 to 10-fold. This increase in radioresistance was a stable, hereditarily established trait, and was preserved for 1.5 - 2 years after isolation of the microorganisms from natural conditions and cultivation on artificial laboratory nutrient media. This observed change in radioresistance applied not only to some one microorganism, but held true for the majority of the isolated cultures.

The facts obtained suggest the possibility of cellular adaptation to ionizing radiations, as to other physical and chemical factors. Our experimental data correspond in principle with the observations of Lozina-Lozinskii and Alexandrov (1959), who, under analogous conditions, discovered a great radioresistance in Paramecium aurellium isolated from the same radioactive waters at the time of our experiments. The adaptation acquired during habitation in a radioactive medium is not, however, as high as the adaptation of microorganisms to chemical substances. The adaptability of a microbial cell to the action of radiation is significantly lower than its adaptation to high concentrations of antibiotics.

The habitat of the radioresistant and the weakly radioresistant cells differed, in a number of cases, in temperature and in salt composition. Thus one might suppose that the higher radioresistance of the microbes, as well as of the paramecium, might have depended to some degree on the thermostability of the cells, if one considers Bellamy and Lewton's observations on some bacteria (1955). An analysis of our data, however, does not connect the observed differences in microbial radioresistance with the chemical composition or the water temperature, since the majority of the most radioresistant cultures were obtained from water sources not differing in the above respects, but different only in their radiation levels. And conversely, differences in radioresistance among microorganisms isolated from weakly radioactive waters (close in their chemical make-up and in temperature to nonradioactive ones) were insignificant. This indicates that the observed differences are primarily determined by the natural radiation level of the medium.

The developmental mechanism of cellular radioresistance, as well as the character of the changes conditioning it, are as yet unclear and require further study. Our data show that the resistance is accompanied by a lowered sensitivity to H_2O_2 , and that it is not connected with drastic changes in the dehydrase, peroxidase, or catalase activity of the cell.

The adaptation process is, apparently, quite complicated and does not depend only on changed conditions of

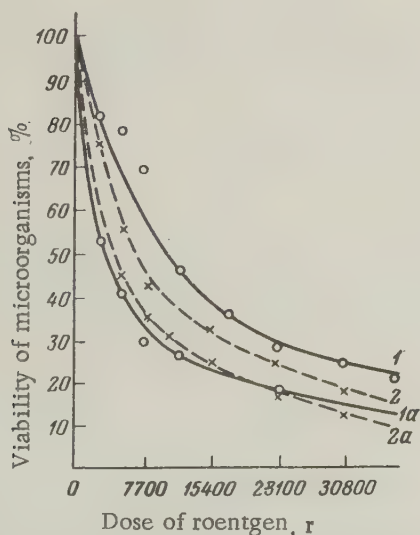


Fig. 2. Survival of microorganisms isolated from weakly radioactive and nonradioactive waters. 1 and 2) M. sulfureus isolated from weakly radioactive waters (30-40 $\mu\text{r/hr}$). 1a and 2a are the same microorganisms isolated from a nonradioactive medium.

peroxide formation in the cell. The absence of increased stability in radioresistant cultures to embichin, which is somewhat similar to radiation in its effects on the organism, merely indicates that the action of these two agents on the cell has more difference than similarity. This difference becomes even more emphasized when cross-adaptations to the above agents are conducted experimentally.

Our conviction that the microbial cell acquires radioresistance by living in increased radiation conditions is now not based solely on the above-presented facts. Currently one of us (Kashkin, 1960, 1960a) has managed to obtain radioresistant cultures by chronic and acute application of various types of irradiation to cells of alkali-formers (the intestinal *Bacterium alcaligenes*)—this in a controlled, clear-cut laboratory experiment. It was possible, in the laboratory, to reproduce artificially the developmental process of cellular radioresistance by subjecting the cell to ionizing radiation—this same process occurs in natural media with increased levels of natural radiation.

SUMMARY

1. Microorganisms isolated from radioactive waters are 3-10 times as radioresistant as similar microorganisms inhabiting nonradioactive water basins. Thus it may be concluded that in principle the cell may acquire resistance to ionizing radiation just as to other outward factors, and that this process takes place in microbes inhabiting media with an increased level of natural radiation.

2. The naturally acquired radioresistance arises in microorganisms only at a fairly high radioactivity level of the medium (362-194 μ r per 1 hour) well above the natural level, concomitantly with an increase in the resistance of the cells to hydrogen peroxide.

3. Radioresistance of microorganisms isolated from a medium with an increased level of natural radiation is a stable hereditary character, since the subcultured radioresistant cultures retained their original properties as long as 1.5-2 years.

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RECOMBINATION IN *Actinomyces aureofaciens*

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At the present time, the use of mutagenic factors is one of the basic methods employed in microbial selection in general, and for the antibiotic producers in particular. As the level of antibacterial activity is increased, however, the number of active strains drops. Apparently this is a general law — the gradual decrease in the number of arising strains with an ever increasing antibacterial activity. For this reason, it seemed timely to work out methods for obtaining hybrids with the aim of using them in selection.

Pontecorvo (1956) and other workers have shown that hybrids (heterozygotic diploids) can be obtained in various types of imperfect fungi.

Later, the same methods were applied by Sermonti (1957) and Sermonti and Spada-Sermonti (1955, 1956) to antibiotic producers of actinomycete origin.

The methods developed for obtaining hybrids were subsequently employed in raising the activity of industrial strains.

Positive results in this connection were achieved for the first time by Sermonti (1957), who crossed two strains of *Penicillium chrysogenum* from divergent lines and obtained a heterozygous diploid whose activity exceeded that of both the original strains. An auxotrophic segregant of this diploid yielded an even higher activity.

Ikeda and Nakamura likewise obtained positive results, employing hybridization in their work with *Aspergillus oryzae* (Ikeda, et al, 1957). They obtained a prototrophic segregant producing 40% more kojic acid than the most active of the original strains.

We used the anastomosis method to obtain an industrial strain of the penicillin producer "New Hybrid" (Alikhanyan and Borisova, 1956; Alikhanyan, et al, 1956).

At present, genetic recombinations have been obtained, likewise, in several species of actinomycetes: *Actinomyces coelicolor*, *Actinomyces fradiae*, *Actinomyces griseoflavus* and *Actinomyces rimosus*. In the latter (i.e., *A. rimosus*), Alikhanyan and Mindlin (1957 a, b), Alikhanyan, et al. (1959), obtained hybrids whose activity considerably exceeded that of the biochemical mutants, reaching the activity level of the original strains and even surpassing it.

As we see from this short survey, the use of hybridization opens up new perspectives for selection in the antibiotic producers.

We should note that, aside from using the direct activity increase in hybrids, a considerably greater change in the hybrid organism as compared to the nonhybrid can be obtained with the subsequent application of mutagens — this was demonstrated by colleagues in our laboratory (Alikhanyan and Mindlin, 1956).

The aim of our work was to study the possibility of hybridization, with its further use for selection, in *Actinomyces aureofaciens*.

The first step in our work was to obtain biochemical mutants. For this, various strains of *A. aureofaciens* (536, Bd., BMK, 11, B-16) were subjected to various mutagens. It is interesting that in obtaining the biochemical mutants we observed a special peculiarity of *A. aureofaciens*: the majority of mutants obtained (99 out of 101) possessed the same requirements, i.e., they needed the addition of arginine for their development. This peculiar tendency to form biochemical mutants identical in their requirements (regardless of the original strain, the mutagen used or the cultivation medium) could serve as a special research theme for the future.

Of the mutants obtained, aside from the arginine ones there were two with other requirements (histidine and isoleucine valine) — these were used to obtain recombination in various combinations with the arginine mutants.

For obtaining hybrid forms we used the usual method of joint inoculation of biochemical mutants, as employed by a number of the above-mentioned authors.

We obtained prototrophs in 17 out of the 18 combinations studied.

Note the relatively high frequency of prototroph occurrence, reaching 3.3% in some combinations (Table 1); we had not observed this with *A. rimosus*.

Morphological Characteristics of the Prototrophs

It is essential to note that the prototrophs obtained from the various combinations (regardless of the original strains) bore a basic morphological resemblance to the classical "wild" *A. aureofaciens*, of which strain No. 536 might serve as an example. Namely, they formed flat mousy-grey or dark grey colonies with aerial mycelia and yellowish-brown substrate mycelia. Some prototrophs differed in their sporulation rate, which in general is not

Combination group	Combination of biochemical mutants	Prototrophs obtained	Prototroph frequency occurrence, %
I	$arg_1^- \times (isl + val)^-$	+	0.13
	$arg_2^- \times (isl + val)^-$	+	0.06
	$arg_3^- \times (isl + val)^-$	+	0.0009
	$arg_4^- \times (isl + val)^-$	+	3.3
	$arg_5^- \times (isl + val)^-$	+	0.0004
	$arg_6^- \times (isl + val)^-$	+	0.13
	$arg_7^- \times (isl + val)^-$	+	0.01
	$arg_8^- \times (isl + val)^-$	+	0.03
	$arg_9^- \times (isl + val)^-$	+	0.006
	$arg_{10}^- \times (isl + val)^-$	—	0.0
	$arg_{11}^- \times (isl + val)^-$	+	0.004
	$arg_{12}^- \times (isl + val)^-$	+	0.02
	$arg_{13}^- \times (isl + val)^-$	+	0.0025
	$arg_{14}^- \times (isl + val)^-$	+	0.0003
II	$arg_3^- \times his^-$	+	0.0015
	$arg_7^- \times his^-$	+	0.0007
III	$his^- \times (isl + val)^-$	+	0.9

a criterion for accurate differentiation. The exception was furnished by the prototrophs of one combination only (arg. $\bar{7}$ x his $\bar{7}$)-they had spores of a dirty beige color and a weak sporulation.

The histidine deficient mutant had a weakly sporulating, light grey, significantly raised compact colony with limited growth. The mutant requiring isoleucine and valine for its growth differed from the original strain only by a less abundant sporulation and a wide asporogenous colony border.

For a more thorough study of segregation, we used the prototrophs of eleven of our obtained combinations, as follows: eight combinations of the first group [arg_1^- through $\text{arg}_8^- \times (\text{isl} + \text{val})^-$]; all the prototrophs of the second group ($\text{arg}_2^- \times \text{his}^-$; $\text{arg}_7^- \times \text{his}$); and all the third group [$\text{his}^- \times (\text{isl} + \text{val})^-$]. In this study we obtained the following results,

All the 29 prototrophs of the eight combinations of group I behaved differently. On the basis of segregation these prototrophs can be divided into six types.

Type I segregation. These prototrophs, in culture, basically resembled the original prototrophs, and segregated one of the latter in very small amounts, namely, the arginine biochemical mutant (0.02 - 0.7%). Segregation in this type corresponded to the following scheme:

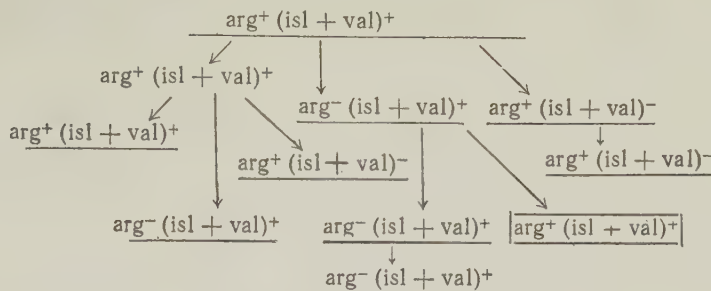
$$\begin{array}{c}
 \text{arg}^+ (\text{isl} + \text{val})^+ \\
 \downarrow \qquad \qquad \downarrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^- (\text{isl} + \text{val})^+ \\
 \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^- (\text{isl} + \text{val})^+ \qquad \text{arg}^+ (\text{isl} + \text{val})^+ \\
 \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^- (\text{isl} + \text{val})^+ \qquad \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^+ (\text{isl} + \text{val})^+ \\
 \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^- (\text{isl} + \text{val})^+ \qquad \text{arg}^+ (\text{isl} + \text{val})^+ \qquad \text{arg}^+ (\text{isl} + \text{val})^+
 \end{array}$$

Type II segregation. A considerably smaller number of prototrophs in the culture segregated the second parent - the isoleucine-valine biochemical mutant - in an amount of $1.5 \pm 0.14\%$, according to the following scheme:

$$\begin{array}{c}
 \text{arg}^+ (\text{isl} + \text{val})^+ \\
 \swarrow \quad \searrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \quad \text{arg}^+ (\text{isl} + \text{val})^- \\
 \swarrow \quad \searrow \quad \downarrow \\
 \text{arg}^+ (\text{isl} + \text{val})^+ \quad \text{arg}^+ (\text{isl} + \text{val})^- \quad \text{arg}^+ (\text{isl} + \text{val})^-
 \end{array}$$

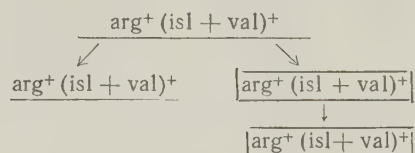
The segregated isoleucine-valine variant did not give rise, in the course of generations, to any other forms.
Type III segregation. The third type comprised a prototroph (it was one out of the 29) which segregated both the original biochemical mutants (1.4 - 0.1%), according to Scheme 3.

Scheme 3. Type III Prototroph Segregation



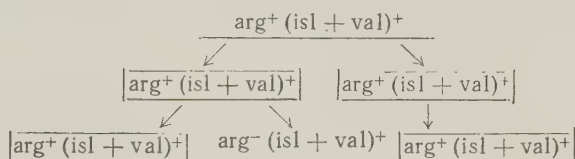
Type IV segregation. Prototrophs of the IV type are presented in Scheme 4, in which we see that the prototroph segregates a small amount (0.15%) of prototrophs, but with a different morphology. Usually these are asporogenous forms resembling the arginine mutant, and sometimes they are colonies with black substrate mycelia of a leathery consistency, with various degrees of sporulation.

Scheme 4. Type IV Prototroph Segregation



Type V prototroph segregation yields, in the first generation, only prototrophs with a different morphology, whereas in the second generation it also segregates the arginine deficient variant (Scheme 5).

Scheme 5. Type V Prototroph Segregation



Type VI segregation. Prototrophs of Type VI proved to be stable, and for several generations did not segregate any other forms.

Quantitatively, all the 29 prototrophs studied could be grouped into six types, as follows (Table 2).

As seen from this table, the greatest number of prototrophs segregated according to Type I, and there was also a considerable number of Type IV and of the non-segregating type.

Note that with one and the same combination we obtained prototrophs segregating according to different types. For example, in the combination $arg_7^- \times (isl + val)^-$, of the five prototrophs studied three were of the first, one of the second, and one was of the sixth type.

Combination Group II, Combining arg^- and his^- Mutants

In this case we studied two combinations, where one and the same histidine variant was matched with different arginine deficient mutants. We studied seven pro-

totrophs for their segregating ability. It proved that they, too, segregated according to some of the above-mentioned types (Table 3).

Just as in the combinations of Group I, the majority of the prototrophs either segregated the arg^- variant, or did not segregate at all, and here likewise, in one and the same combination we observed prototrophs differing in their segregation type.

Combination Group III, where the histidine deficient mutant was paired with the isoleucine-valine mutant, was represented by one combination.

Among the five prototrophs of this combination studied, four were of the sixth and one was of the third type, i.e., it segregated both the original biochemical mutants (in an amount of 0.045%) (Table 4).

Antibacterial Activity of the Prototrophs

We studied the antibacterial activity of 110 prototroph colonies. As controls we used the biochemical mutants, and the original strains from which the biochem-

TABLE 2. Quantitative Distribution of Group I Prototrophs [of the $\text{arg}^- \times (\text{isl}+\text{val})^-$ combination] by Segregation Types

Types of prototroph segregation	Forms segregated by the prototrophs	No. of original prototrophs belonging to the given type
I	a) Prototrophs b) One of the original biochemical mutants	16
II	a) Prototrophs b) The second original biochemical mutant	3
III	a) Prototrophs b) Both original biochemical mutants	1
IV	a) Prototrophs b) Prototrophs with changed morphology	4
V	a) Prototrophs b) Prototrophs with changed morphology c) One original biochemical mutant (in the second generation)	1
VI	a) Prototrophs	4

TABLE 3. Quantitative Distribution of Group II Prototrophs (of the $\text{arg}^- \times \text{his}^-$ combination) by Segregation Types

Types of prototroph segregation	Forms segregated by the prototrophs	Number of prototrophs
I	a) Prototrophs b) One of the original biochemical mutants	2
II	a) Prototrophs b) The second original biochemical mutant	1
IV	a) Prototrophs b) Prototrophs with changed morphology	1
VI	a) Prototrophs	3

TABLE 4. Quantitative Distribution of Group III Prototrophs $[(\text{isl}+\text{val})^- \times \text{his}^-]$ combination] by Segregation Types

Types of prototroph segregation	Forms segregated by the prototrophs	Number of prototrophs
III	a) Prototrophs b) Both original biochemical mutants	1
IV	a) Prototrophs	4

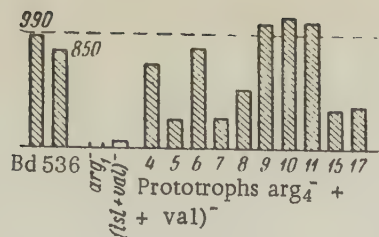


Fig. 1. Activity of original strains, biochemical mutants and ten prototrophs of the combination $arg_4^- \times (isl + val)^-$.

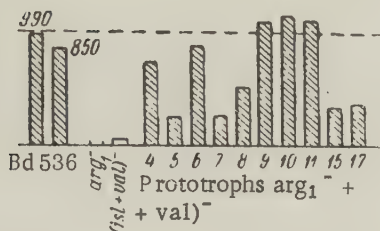


Fig. 2. Activity of original strains, biochemical mutants and ten prototrophs of the combination $arg_1^- \times (isl + val)^-$.

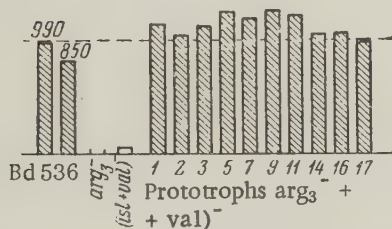


Fig. 3. Activity of original strains, biochemical mutants and prototrophs of the combination $arg_3^- \times (isl + val)^-$.

ical mutants were derived. The activity of biochemical mutants did not exceed 100 units/ml; in the arginine mutants it was frequently nil. All the prototrophs greatly exceeded the biochemical mutants in antibacterial activity, attained the levels of the original strains, and in some cases exceeded even those by 5-20%. As an example, the diagrams present the activity of the prototrophs of three combinations with ten prototrophs each [$arg_1^- \times (isl + val)^-$; $arg_4^- \times (isl + val)^-$; $arg_3^- \times (isl + val)^-$] (see Figs. 1-3).

As in the combination $arg_4^- \times (isl + val)^-$, presented in Fig. 1, the prototrophs in the combinations $arg_2^- \times (isl + val)^-$; $arg_7^- \times (isl + val)^-$; $arg_8^- \times (isl + val)^-$; $arg_5^- \times (isl + val)^-$; $arg_7^- \times his^-$; $his^- \times (isl + val)^-$ did not exceed the original strains in activity.

In the combinations $arg^- \times (isl + val)^-$ and also $arg^- \times his^-$, some prototrophs did exceed the activity

of the original strains, as was also true for the combination $arg_1^- \times (isl + val)^-$ (Fig. 2).

Figure 3 shows the activity of the prototrophs of the $arg_3^- \times (isl + val)^-$ combination—the only one in which all the prototrophs equaled or exceeded the levels of the original strains in their antibacterial activity.

As we see from these diagrams, all the prototrophs of the $arg^- \times (isl + val)^-$ combinations had a lower activity than the original strains; in the $arg_1^- \times (isl + val)^-$ combinations, only a few prototrophs possessed an activity exceeding the original strain; whereas in the last, the $arg_3^- \times (isl + val)^-$ combinations, almost all the prototrophs exceeded the activity of the original strains by 5-20%. It is of interest to note that one and the same isoleucine-valine mutant participated in these combinations—the mutant was obtained from strain 536; the three arginine mutants likewise came from one and the same strain, Bd, and did not differ from one another either morphologically or in antibacterial activity. It is probable that, alongside the arginine deficiency, each of these mutants possessed specific physiological traits which conditioned the differences in antibacterial activity in the prototrophs of the above-mentioned combinations.

We classified the prototrophs by activity, and chose the most active one, [$arg_3^- \times (isl + val)^-$]—II, which we propose to use in studying the scope of induced variation in the prototrophs as compared to the original strains.

We also studied the antibacterial activity of prototrophic segregants. Activity in the non-sporulating prototrophs, which morphologically resemble the arg^- biochemical mutants, is usually nil, whereas in the segregants, e.g., [$arg^- \times (isl + val)^-$]—3 with the black substrate mycelium, it is in the range of 300 units/ml.

SUMMARY

1. In *Actinomyces aureofaciens* there occur recombinants, as in *A. rimosus*, but unlike the latter the prototrophs obtained in *A. aureofaciens* are morphologically similar to the "wild" type.

2. *A. aureofaciens* prototrophs vary in the character of segregation and segregate one or both of the original biochemical mutants and eventually prototroph segregants morphologically different from the prototrophs, which never occurs in the prototrophs of *A. rimosus*.

3. The antibacterial activity of all prototrophs is many times higher than that of the biochemical mutants attaining the level of the initial strains and eventually even exceeding it by 5-20%. There is some reason therefore to suggest the diploid nature of the recombinants.

4. The recombinants of *A. aureofaciens* can be used for producing new, highly active industrial strains.

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EFFECT OF THE ACIDITY OF THE MEDIUM ON THE RATIO OF FERMENTATION PRODUCTS OF *Lactobacterium pentoaceticum*

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The lactic acid bacteria which are combined into the heterofermentative group vary greatly in the physiological respect. The chemism of the fermentation is not entirely clear. However, observations show (Shaposhnikov and Semenova, 1949; Rabotnova, 1957; Tarasova, 1958) that this fermentation can shift markedly through the effect of external factors. In the present work, it was determined that shifts in the ratio of products of heterofermentative lactic acid fermentation by *Lactobacterium pentoaceticum* isolated from kvass wort can be caused by a change in the pH of the medium.

METHOD

In the work, 5.5 Bwort + MnSO_4 (110 mg per 1 liter) was used. The experiments were carried out in ordinary test tubes containing 35 ml of medium. A 24-hr culture from wort with pellet and chalk served as the inoculum for experiments in which the acids produced were neutralized with CaCO_3 and MgCO_3 . For media without neutralization, the culture was first passed through an experimental medium before being used as inoculum in the experiments. Inoculations were made with a 1% culture (initial number of cells in the medium — 20-30 million per 1 ml). The temperature of cultivation was 37°C. The methods for determining the sugar consumed, the fermentation products, and the crop of bacteria have been described by us earlier (1961). Bacterial carbon and total wort carbon in experiments without neutralization were determined by means of wet combustion (Sorokin, 1954). Prior to the determination of carbon, concentrated H_3PO_4 was added to the fermentation mixture in order to dissolve protein flocs precipitating as the bacteria developed. After this, the fermentation mixture was centrifuged, and the cells were washed with distilled water and combusted.

All products were expressed in millimoles per liter, sugar was expressed in millimoles of trioses per liter, while the bacterial crop was expressed in millions per ml. Average results of two or several parallel experiments are given after control determinations in sterile wort were subtracted. Each experiment was carried out in duplicate.

EXPERIMENTAL RESULTS

Determination of the pH limits at which the growth of *L. pentoaceticum* is possible

The effect of the initial pH of the medium on the dynamics of growth of *L. pentoaceticum* was investigated. Initial pH's of from 2.77 to 8.56 were produced following sterilization of the wort using sterile solutions of H_3PO_4 and NaOH.

The results presented in Fig. 1 show that growth of *L. pentoaceticum* was possible in a wide pH range — from 3.8 to 8.5 — but the extent of growth within this range varied. The higher the initial pH, the greater was the cell crop. However, at pH 8.56 there was a long lag at the start of growth, although the final crop was the greatest (Fig. 1a). It is seen from the data presented in Fig. 2 that, as the bacteria grew, they rapidly and strongly acidified the medium to the same level regardless of the initial pH value.

Ratio of the fermentation products when the medium has a slightly acid reaction

In order to determine how the pH affects the character of the *L. pentoaceticum* fermentation, the fermentation was investigated in the dynamics of growth of the culture in naturally acidified wort in which a pH of 3.2-3.4 was rapidly established, and in wort where the pH was maintained at a higher level during the course of the fermentation. Phosphate buffers added to the wort which had previously been neutralized to pH 8 and 6.5 did not maintain these pH values due to intensive acid production in the *L. pentoaceticum* culture. Therefore, in order to neutralize the acids produced, small portions of CaCO_3 , MgCO_3 , MgO and CaO were added to the fermentation mixture during the course of growth of the culture. The medium was thoroughly mixed from time to time. MgO and CaO produced a pH higher than 10, preventing the growth of the bacteria. In the presence of chalk, considerable acidification was observed during the first day of fermentation, but then the acidity became stabilized at about 5.0, i.e., 1.5 pH units higher than without chalk. This pH value proved to be exceptionally favorable for the bacteria. The maximal num-



Fig. 1. Dependence of growth (max) of *L. pentoaceticum* on the initial pH of the medium.

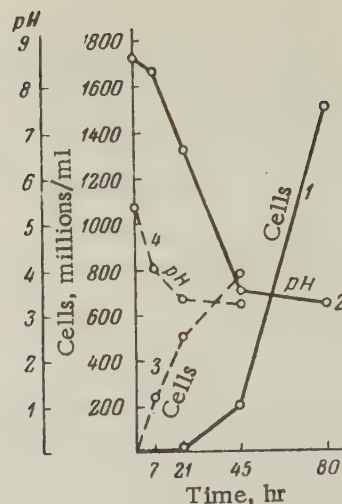


Fig. 1a. Dynamics of growth of *L. pentoaceticum* in wort. 1) Number of cells; 2) pH when initial pH is 8.56; 3) number of cells; 4) pH when initial pH is 5.3.

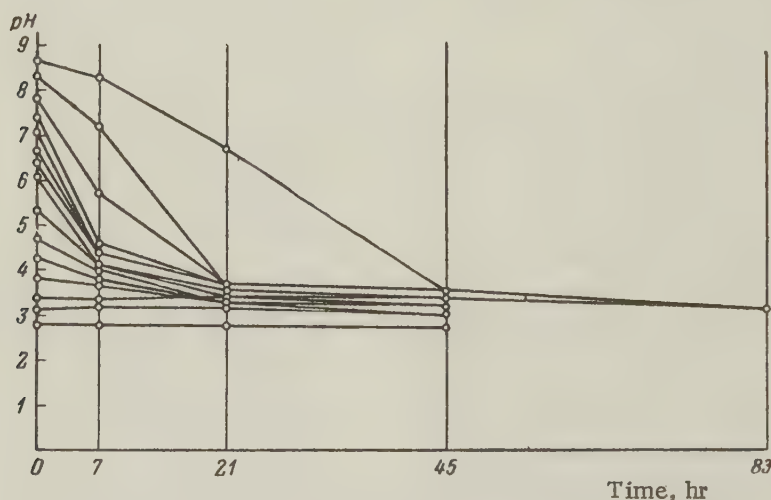


Fig. 2. Dynamics of pH changes in an *L. pentoaceticum* culture in wort with various initial pH values.

ber of cells under these conditions exceeded 3 billion/ml. The large crop was accompanied by a high rate of utilization of sugar and a high yield of products. The principal fermentation products of *L. pentoaceticum* when wort sugars were utilized were alcohol and lactic acid. Acetic was produced in very small amounts. Carbon dioxide was also produced, but it was impossible to measure its amount in the fermentation with chalk because carbon dioxide was simultaneously produced from the chalk as the result of its reaction with the acids being formed. Thus, the fermentation of the organism isolated by us was a *Leuconostoc mesenteroides* type

fermentation. According to the ratio of products, it differed from the other type of heterofermentative lactic acid fermentation, where the principal products are lactic and acetic acids (Shaposhnikov and Semenova, 1949; Tarasova, 1958).

The ratio of products changed somewhat during the course of fermentation (Fig. 3). By the time of maximal growth of the bacteria (first 24 hr), the ratio lactic acid/alcohol was 1/1. After 24 hr, the rate of alcohol production decreased noticeably, and lactic acid became the principal fermentation product. After 72 hr, the ratio lactic acid/alcohol was 3/2.

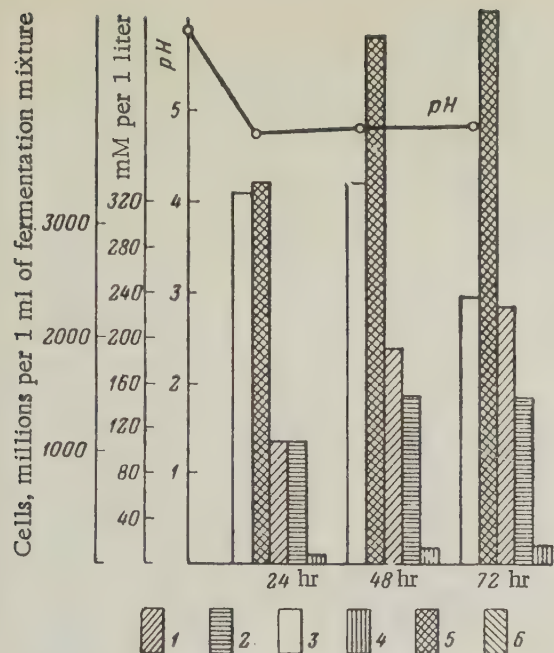


Fig. 3. Dynamics of fermentation in slightly acid medium (wort with chalk). 1) Lactic acid; 2) alcohol; 3) number of cells; 4) acetic acid; 5) sugar as trioses; 6) CO₂.

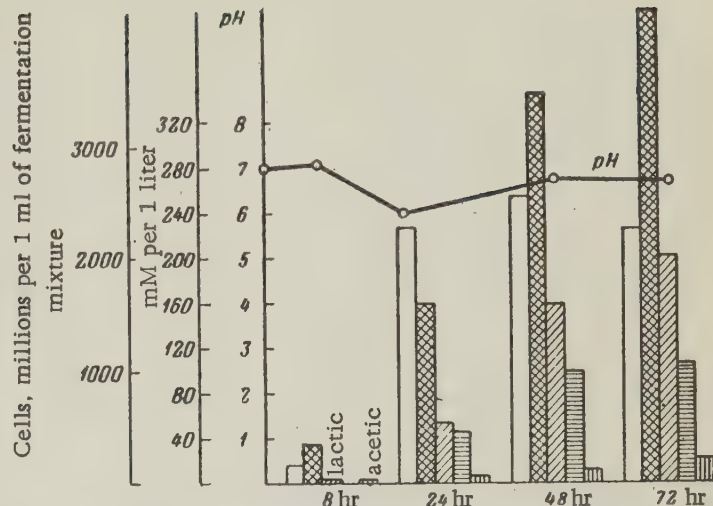


Fig. 4. Dynamics of fermentation under neutral conditions (wort with MgCO₃). The designations in Figs. 4 to 7 are the same as in Fig. 3.

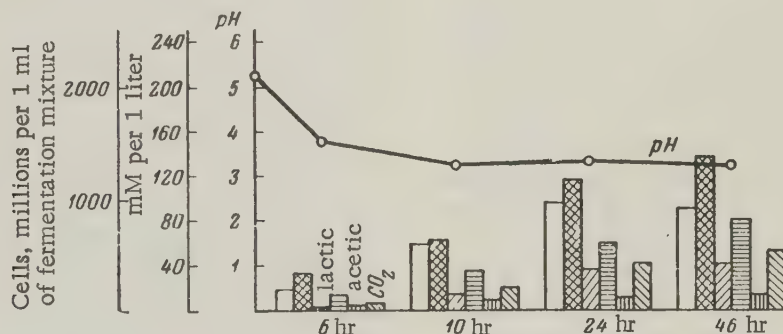


Fig. 5. Dynamics of fermentation in strongly acid wort.

Ratio of fermentation products when the reaction of the medium is neutral

MgCO₃ maintains the pH of the fermentation mixture at about 7.0—nearly 2 units higher than chalk. Under these conditions, the maximal crop reached 2.5 billion/ml, i.e., it was somewhat lower than with chalk. Nevertheless, a neutral pH can be considered entirely favorable for the bacteria, since the general level of growth and fermentation was very high. The ratio of products under neutral conditions changed in comparison with fermentation on wort with chalk (Fig. 4). The yield of acetic acid increased slightly. The proportion of the neutral product — alcohol — decreased with respect to lactic acid. After 24 hr, the ratio lactic acid/alcohol was 1.2, but by the end of fermentation (72 hr) this ratio increased to 2, i.e., exceeded that observed in slight-

ly acid medium. As in the case of the fermentation with chalk, lactic acid was the principal fermentation product.

Ratio of fermentation products when the reaction of the medium is strongly acid (initial pH 5.0 - 5.5)

In wort without neutralization, the pH dropped to 3.2-3.4 as early as the 10th hour, and remained at about that level throughout the entire fermentation. This acidity proved to be unfavorable for the bacteria (Fig. 5). Growth and fermentation were strongly inhibited. The maximal cell crop did not exceed 1 billion/ml. A great deal of unfermented sugar remained. The ratio of the products changed markedly. Whereas the yield of acetic acid did not decrease as compared with fermentation in the presence of chalk, the amount of lactic acid was

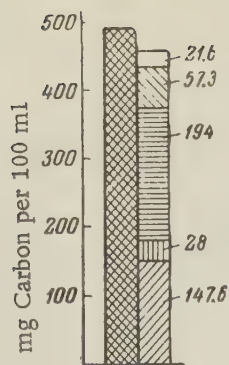


Fig. 6. Carbon balance of fermentation in strongly acid wort.

greatly reduced. Alcohol production was relatively less depressed. It became the principal fermentation product. The accumulation of acids essentially ceased after 24 hr, while alcohol production continued even after 24 hr. By the end of the experiment (46 hr), the ratio lactic acid/alcohol was 0.5. The predominant accumulation of alcohol under these conditions was probably in the nature of a reaction of the organism in response to the acid pH of the medium. It was as if by increasing the yield of the neutral product which does not alter the pH the bacteria were protecting themselves from excessive acidity. As a consequence of this, there was a ratio of products to the sugar utilized which was entirely unusual for heterofermentative lactic acid fermentation: for 1 mM of hexose consumed, there were nearly 1.2 mM of alcohol and only about 0.75 mM of lactic acid. The question arises of whether all of the fermentation products are formed from sugar under strongly acid conditions, or whether part of them are synthesized from other components or wort. In order to answer this question, we calculated the carbon balance of the fermentation. Wort may contain hexoses, pentoses, and up to 3-4% pentosans, which give pentoses upon hydrolysis (Leberle and Schuster, 1956). We did a chromatographic analysis of different batches of wort according to the method employed by Rylkin (1959). The analysis showed that wort always contains raffinose, sucrose, maltose, glucose and fructose, but practically no pentoses, either before or after hydrolysis of the wort. This entitled us to express the carbon of the sugar consumed by the bacteria in milligrams of hexose C (or $2C_3$). As shown by Fig. 6, in naturally acidified wort, the carbon skeleton of the bacteria and the fermentation products can be formed at the expense of C_6 or $n-C_6$ sugar. Figure 5 presents data of the dynamics of the same experiment. The data pertain to 46 hr of fermentation. A comparison of the balance data and the unusual ratio of

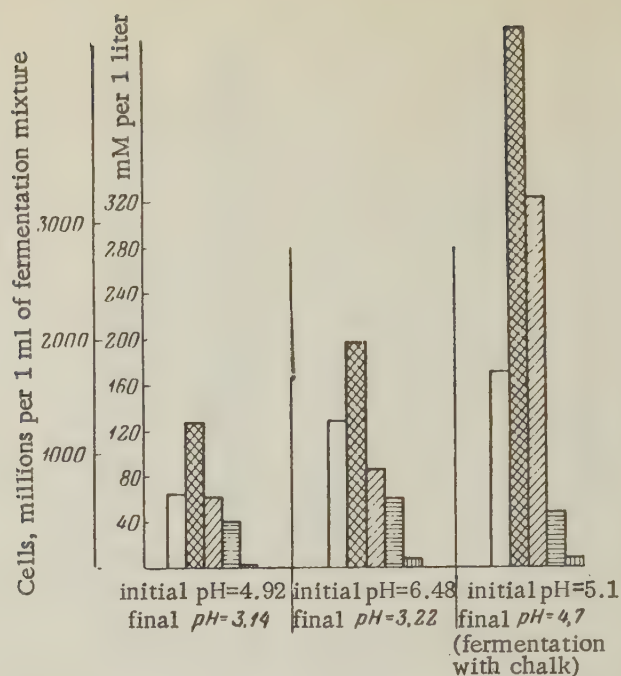


Fig. 7. Ratio of fermentation products on medium with peptone.

products and sugar forces one to assume that, in strongly acid wort, the formation of fermentation products may proceed by a pathway different from that in media containing $MgCO_3$ and $CaCO_3$, i.e., under conditions of more favorable acidity.

In order to determine whether the high accumulation of alcohol in acid conditions depends on the composition of the nutrient medium, a fermentation was carried out in which the biomass crop and the yield of end products of the fermentation were measured in peptone-containing medium, where the ratios and composition of the components were different from those in wort (in %): glucose - 5; peptone (Difco) - 1; K_2HPO_4 - 0.05; KH_2PO_4 - 0.05; $MgSO_4$ - 0.02; $MnSO_4$ - 0.01; yeast autolyate - 0.5.

Unfortunately, we were unable to use media of simpler and more definite composition, because we did not get satisfactory growth of the bacteria on them. The results presented in Fig. 7 show that the initial pH altered the fermentation on peptone primarily in the quantitative respect. Neutralization with chalk greatly increased the cell crop and the yield of lactic acid. However, even under conditions of low pH lactic acid remained the major fermentation product, although the relative amount of alcohol did increase. This shows that the production of alcohol in strongly acid medium depends not only on the pH, but on the composition of the nutrient medium as well.

DISCUSSION OF RESULTS

The investigations conducted showed that *L. pentosaceticum* grows within a rather wide pH range - from 3.8 to 8.5. Within these limits, the higher the initial

pH, the better the growth. Many microorganisms can regulate the pH of the medium by releasing acid, neutral, or alkaline metabolic products, in this way creating conditions of acidity which are suitable for their growth or preventing a further shift in the pH in the unfavorable direction. *L. pentoaceticum* does not regulate the pH of the medium. Regardless of the initial pH value, the bacteria rapidly and strongly acidify the medium to the same level as they grow, in this way creating conditions which are unfavorable for their own growth. However, analysis of the fermentation products shows that the bacteria nevertheless alter their metabolism in relation to the pH, adjusting it to a certain acidity and in some measure moderating the unfavorable effect of this factor.

Slightly acid conditions (pH of about 5) are optimum for growth. Neutral conditions are not entirely optimum. Some acidification of the medium is necessary for the start of active reproduction at a high pH. At pH 8.56, the culture introduced into the medium lowers the pH noticeably even before the start of rapid multiplication, and only after this does the number of cells increase greatly. Under these conditions, the lag phase is especially long.

Under optimal conditions of acidity, the ratio of products found is entirely usual for the heterofermentation: by the time of maximal bacterial growth, alcohol and lactic acid are produced in equimolar amounts. Under neutral conditions, the ratio of products changes. The yield of acetic acid increases, and the amount of alcohol decreases. It is as though the bacteria endeavor to shift the pH to the optimal side by producing more acids. In naturally acidified wort (initial pH about 5), the pH becomes stabilized at the level of 3.2-3.4 from the first hours of fermentation. These conditions are so unfavorable for the bacteria that growth and fermentation are greatly depressed, but the ratio of products is not entirely usual for heterofermentative lactic acid fermentation. The neutral product — alcohol — becomes the major product during the course of the entire fermentation. It is well known that in many fermentations the production of neutral products is a measure of the self-defense of the bacteria against extreme acidity. Therefore, an acid pH of the medium is often a necessary condition for their accumulation (Ierusalimskii, 1942; Orlova, 1950; Rabotnova, 1957). Consequently, the predominant production of alcohol in the *L. pentoaceticum* fermentation in strongly acid wort can be regarded as a means by which this organism reacts against strong acidity. The higher the pH, the less alcohol is produced in the fermentation. Its production is of a clearly adaptive character and depends on the pH and composition of the nutrient medium. In peptone-containing medium, even under conditions of very acid pH, lactic acid remains as the principal fermentation product, although the relative amount of alcohol increases as the acidity rises. The carbon skeleton of all products in naturally acidified wort is appar-

ently formed from C_6 and $n-C_6$ sugar. This is confirmed by the carbon balance of the fermentation.

When the balance data and the unusual ratio of products are compared, it can be assumed that the formation of fermentation products in strongly acid wort may proceed by a pathway different from that when the fermentation mixture is neutralized, i.e., under conditions of favorable acidity. The mechanism of the heterofermentative lactic acid fermentation by *L. pentoaceticum* is evidently not identical in all cases, but is determined by the conditions of cultivation.

I should like to offer my sincere thanks to Prof. I. L. Rabotnova, the director of the work, as well as to Prof. V. N. Shaposhnikov, for valuable advice and guidance.

SUMMARY

1. The growth of the heterofermentative lactic acid bacterium *L. pentoaceticum* is possible within the pH range of 3.8 to 8.5.

2. *L. pentoaceticum* rapidly and strongly acidifies the medium to pH 3.2-3.4 as it grows, regardless of the initial pH value.

3. In wort with chalk, where the pH is maintained at about 5.0 during the course of the fermentation, an optimal crop of bacteria of 3.5 billion cells per ml is reached. The principal fermentation product is lactic acid. After 72 hr, the ratio lactic acid/alcohol is 3/2.

4. In wort with $MgCO_3$ (pH about 7), the maximal cell crop is 2.5 billion per 1 ml. The yield of acetic acid increases, while the alcohol yield decreases. After 72 hr, the ratio lactic acid/alcohol is 2. Lactic acid remains the principal fermentation product.

5. In strongly acid wort, growth and fermentation are depressed. The ratio of products changes markedly. Alcohol is found to be the principal product. After 46 hr, the ratio lactic acid/alcohol is 1/2.

6. In a peptone-containing medium, changes in the initial pH do not cause such profound shifts in the ratio of the fermentation products of *L. pentoaceticum*. Even with a very acid pH, lactic acid remains as the principal fermentation product on peptone, although the relative amount of alcohol does increase.

7. The predominant accumulation of alcohol in strongly acid wort should be regarded as the means by which *L. pentoaceticum* reacts against high acidity of the medium. Its production is of an adaptive character, and depends on the pH and the composition of the nutrient medium.

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EFFECT OF THE PARTIAL INHIBITION OF RESPIRATION IN THE FUNGUS *Aspergillus oryzae* ON THE SYNTHESIS OF BIOMASS, P₇ CONTENT, AND AMYLOLYTIC ACTIVITY

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Increased enzymatic activity in plants is of definite theoretical and practical interest. Recent investigations have established (Malkov et al., 1955; Deeva, 1958) that the partial inhibition of the respiration of certain seeds by means of such substances as NaN₃, KCN, NaF, K₂S, KH₂PO₄, SO₂, and others leads to the production of malt with a higher amylolytic and proteolytic activity as compared with the control. The aim of the present work was to study the effect of certain inhibitors on *Aspergillus oryzae*, because it, in the form of a fungal enzyme preparation (fungal malt), has found wide application in the alcohol, beer brewing, and other branches of the food industry (Kalashnikov and Lifshits, 1955; Vyatkin, 1958).

METHODS

The experiments were carried out with a culture of *A. oryzae* (strain 476). The fungus was grown on laboratory shakers in flat-bottomed 500 ml flasks containing 100 ml of medium, for three days at 28-30 C. Inoculations were made with a suspension of conidia washed off from the surface of wort agar for each experiment, and calculated to give one million conidia per 1 ml of medium. Liquid defined medium of the following composition (in %) served as the nutrient substrate: soluble starch-6; (NH₄)₂SO₄-0.05 (by nitrogen); KCl-0.05; MgSO₄-0.05; K₂HPO₄-0.1; FeSO₄-0.001; CaCO₃-0.1. According to existing observations (Smirnova, 1956), the given medium favors the passage of amylolytic enzymes from the mycelium to the culture fluid.

The chemical compounds, the effect of which was studied, were added to the nutrient medium in the form of concentrated solutions calculated to give the required concentrations in the final dilution; after this, the conidial suspension was added. The volumes of medium in the flasks were equalized when necessary by adding sterile water. At the end of the experiments, the mycelium was filtered off with suction on a Buchner funnel, washed, and weighed. The moisture of the mycelium was determined by drying it to constant weight; the amount of easily hydrolyzable phosphorus was established after grinding weighed portions with quartz sand and subse-

quent precipitation of the protein with trichloroacetic acid, filtration, and hydrolysis of the protein-free filtrate for 7 minutes with a 1 N solution of HCl at 100 C and colorimetric measurement of the hydrolyzate obtained according to the method of Abagal'yants and Dubrovskaya (Frolov-Bagreev and Abagal'yants, 1951); oxygen consumption was determined in Warburg respirometers in the same culture fluid in which the fungus had been grown. In some experiments, after the nutrient medium was inoculated with conidia in the presence or absence of the chemical compounds being tested, part of it was placed in the manometer vessels where the fungus was grown in the same manner as in the main flasks. After three days of growth, 5% alkali was added to the center well, the manometer was attached, and the extent of oxygen consumption was measured by the usual method. The activity of α -amylase was determined by its activity on α -amylodextrin (Belozerskii and Proskuryakov, 1951). The conditional units of α -amylase, expressed as the number of grams of soluble starch dextrinized in an excess of β -amylase by 100 ml of culture fluid in 1 hr at 30 C, were calculated on the basis of time of dextrinization and the amount of test fluid used for the experiment. In certain experiments, total amylolytic activity was determined by the amount of starch converted to maltose in 1 hr at 30 C; calculations were made in the same way as in the determination of α -amylase activity.

EXPERIMENTAL

Experiments with sodium fluoride

It is known that fluoride is an enzyme poison which, according to the accepted view, acts on enolase; as the result of this, during the conversion of sugar, the transition of 2-phosphoglyceric acid to phosphoenolpyruvic is uncoupled. Keeping in mind that the uncoupling of the process of pyruvic acid production is connected, under any circumstances, with the cessation not only of anoxybiotic, but with the oxybiotic transformation of sugar as well, the action of sodium fluoride should lead to the complete cessation of the vital activity of the organisms. However, experiments carried out with yeasts (Malkov

and Bogdanova, 1956) showed that sodium fluoride in a concentration of 0.0001 M did not inhibit alcoholic fermentation and reproduction of the yeast at all, and at higher concentrations it caused only partial inhibition.

The first experiments of the present investigation, carried out with the addition of NaF to the nutrient medium (Table 1), showed that the NaF concentration at which the synthesis of the fungal biomass did not decrease in comparison with the control was about 0.01 M. Lower NaF concentrations even had a stimulatory effect on this process in a number of cases. As for amylolytic activity, the cultivation of the fungus under aerobic conditions in the presence of 0.01-0.0025 M NaF promoted considerable activation of its amylolytic activity, as seen from the data presented in Tables 1 and 2.

Inasmuch as it had been noted in our investigations (Malkov et al., 1955; Deeva, 1956, 1958) that the partial inhibition of the respiration of the plant organism is accompanied by an increase in the activity of certain hydrolytic enzymes, we had to determine what effect NaF in concentrations increasing the ability of the fungus to produce amylolytic enzymes would have on the intensity of its respiration as well as on its ability to

accumulate energy-rich phosphates. It was also of some interest to check how the inhibition of respiration of the fungus would reflect on the efficiency of its utilization of the carbohydrates in the medium. The results obtained are given in Table 3.

As seen from the data presented, the presence of NaF in the nutrient medium caused a reduction in the respiration rate of the fungus on the one hand, and promoted the accumulation of energy-rich phosphates on the other. When the efficiency of the utilization of the carbohydrates of the medium by the fungus was tested, it was found that the inhibition of respiration was accompanied by an increased yield of mycelium per unit of carbohydrate assimilated.

The data presented showed that during the cultivation of *A. oryzae* fluoride in the concentration of 0.001-0.002 M, first of all partially inhibited respiration (by 31.9% under the conditions of the last experiment); second, partial inhibition of respiration of the fungus promoted the synthesis of biomass and an increase in the content of energy-rich phosphates in the mycelium; and third, the partial inhibition of respiration promoted a considerable increase in its α -amylolytic activity.

TABLE 1. Effect of Various NaF Concentrations on the Synthesis of Fungal Biomass and its α -Amylase Activity

Experiment	Concentration of NaF, M	Dry weight of mycelium in g	α -Amylase activity in		pH of medium after growth
			conditional units	% of control	
I	0	2.06	4800	100.0	-
	0.002	2.80	8000	166.7	-
	0.005	2.10	2400	50.0	-
	0.01	2.10	3430	71.5	-
II	0	2.10	2000	100.0	7.21
	0.001	1.92	4000	200.0	7.12
	0.0025	2.23	1500	75.0	7.32
	0.005	2.10	1200	60.0	7.24
	0.01	2.03	1000	50.0	7.40

TABLE 2. Effect of NaF on the Amylolytic Activity of the Fungus

Experiment	Concentration of NaF, M	Amylolytic activity in g of starch per 1 hr at 30 C			
		total in		α -amylase in	
		conditional units	% of control	conditional units	% of control
I	0	40.85	100.0	2182	100.0
	0.002	54.20	132.7	3050	139.8
II	0	29.90	100.0	3500	100.0
	0.002	40.65	136.0	4800	137.4

Experiments with Sodium Azide

According to existing views, sodium azide is a poison which inhibits the activity of iron-containing oxidases. Some observations showed (Malkov and Leoninok, 1959) that sodium azide in the concentration of 0.001 M inhibits the process of fermentation considerably and completely uncouples respiration in yeast. It was interesting to check what effect this inhibitor would have on the physiological activity of *A. oryzae*.

The experiments presented in Table 4 showed that sodium azide inhibited the reproduction process of the fungus. This inhibitory effect of sodium azide which was slightly noticeable at a concentration of 0.0001 M increased when the concentration of the poison was raised. As for amylolytic activity, this inhibitor in a concentration of 0.001 M strongly activated the production of amylases by the fungus; high concentrations caused the reverse effect, however. Sodium azide, as was the case with fluoride, increased acid production by the fungus somewhat, judging by the lowering of the pH of the medium toward the end of the fermentation, and also caused a partial inhibition of the mycelial respiration. In all experiments, with the exception of those with sodium azide, the inhibition of respiration of the vegetative organism usually led to an increase in its content of energy-rich phosphates. The exception to the general rule which was observed (Table 4) can be explained by the toxic effect of sodium azide, which was not limited to the inhibition of the activity of one or another enzyme, but spread to the cell protoplasm. This is largely confirmed by systematic observations according to which sodium azide, even in minute concentrations, inhibited the synthesis of the fungal biomass.

TABLE 3. Effect of Various Concentrations of NaF on the Synthesis of Fungal Biomass, its α -Amylase Activity, Respiration Rate, P_7 Content of the Mycelium, and Efficiency Coefficient

Experiment	NaF concentration, M	Weight of mycelium,g	α -Amylase activity in		P ₇ per dry material in		Respiration as O ₂ consumed in mm ³ in 10 minutes		pH at end of experiment	Efficiency coefficient
			conditional units	in % of control	mg%	% of control	per 1 g of dry material	in % of control		
I	Control	2.49	1200	100.0	200	100	26.9*	100.0	7.75	-
	0,001	2.51	1500	125.0	248	124	25,3*	94.1	-	-
	0,0025	2.08	1300	108.3	213	106	14,5*	53.9	6,91	-
II	Control	2.67	1140	100.0	132	100	210.0	100.0	-	0.89
	0,001	3.01	4500	394.7	173	131	143.0	68.1	-	1.23
	0,002	2.34	5400	473.7	153	116	-	-	-	1.00

* Growth of mycelium in manometer vessel; data on respiration pertain to 1 ml of medium.

TABLE 4. Effect of Various Concentrations of NaN_3 on the Synthesis of Biomass, Amylolytic Activity, Respiration, and the Content of Energy-Rich Phosphates (P_7) in the Mycelium

Experi- ment.	NaN ₃ concen- tration, M	Dry weight of mycelium,g	α-Amylase activity in		P ₇ per dry ma- terial in		Respiration as O ₂ consumed in 10 min., in mm ³		pH at end of experi- ment
			condition- al units	% of control	mg %	% of control	per l ml of medium	in % of control	
I	Control	2,06	2000	100.0	-	-	-	-	7.29
	0.0001	1,94	4000	200.0	-	-	-	-	7.01
	0.001	1,75	1710	85.5	-	-	-	-	7.16
	0.002	No growth							
II	Control	2,49	-	-	200	100	26.91*	100	7.75
	0.0001	2,46	-	-	186	93	14.76*	55	6.72
III	Control	2,07	1477	100.0	136	100	54.07*	100	-
	0.0002	2,00	1920	130.0	112	82	41.77*	77	-
	0.0004	1,88	1226	84.3	113	83	-	-	-

* Growth in manometer vessels.

TABLE 5. Effect of Sodium Azide on the Respiration of the Fungus, its Content of Energy-Rich Phosphates, and Amylolytic Activity in the Presence of Ferrous Ions (Fe^{++})

Experimental variant	Dry weight of mycelium in g	α -Amylase activity in		P_7 per dry material in		Respiration as O_2 consumed in 10 min, in mm^3	
		conditional units	% of control	mg %	% of control	per 1 g of dry mycelium	in % of control
Control (without NaN_3)	2.41	1370	100.0	325	100.0	375.0	100.0
0.0002 M NaN_3	1.94	1600	116.9	306	94.1	37.5	10.0
Per 100 ml of medium + 5 mg of Fe^{++}	2.11	960	70.1	-	-	398.7	106.2
Per 100 ml of medium + 5 mg of Fe^{++} and 0.0002 M NaN_3	1.77	1600	116.9	385	118.4	27.3	7.3

The question of the formation and breakdown of energy-rich phosphates during the course of cultivation of the fungus in the presence of sodium azide was also examined in the experiment presented in Table 5 where, in distinction from preceding experiments, aside from the independent effect of sodium azide on the physiology of the fungus, we also studied the effect of the presence of ferrous ions in the medium which, as is known, are activators of oxidative processes.

The data presented in Table 5 show that sodium azide inhibits respiration and the synthesis of biomass regardless of the presence of a supplementary amount of iron in the medium. The inhibitory effect of sodium azide on respiration in the case where excess iron was added immediately led to an increase in the amyolytic activity and an increase in the P_7 content of the mycelium.

Experiments with Diethyldithiocarbamate

It is known that the respiration of the fungus is ensured not only by the cytochrome-cytochrome oxidase system, but by other oxidative systems as well. Among the latter is the polyphenol oxidase enzyme system, for which diethyldithiocarbamate (DDC) is a specific inhibitor (Kubowitz, 1937). In order to determine the ex-

tent of the participation of iron- and copper-containing oxidative systems in the respiration of the fungus in the process of its vital activity, in one case sodium azide in a concentration of 0.0002 M was added to the nutrient medium, but in another case diethyldithiocarbamate was added in the same concentration.

The data presented in Table 6 show that the respiration of the fungus occurs chiefly with the participation of an iron-containing respiratory system. The strong inhibitory effect on the respiration of the fungus in the presence of sodium azide is due to the circumstance that sodium azide is a more universal respiratory inhibitor than is diethyldithiocarbamate. As is well known, sodium azide acts in an inhibitory manner on the activity of many iron- and copper-containing oxidases, whereas diethyldithiocarbamate acts predominantly on copper-containing oxidases, which are apparently represented to a much lesser extent in the fungal mycelium. At the same time, however, the partial inhibition of the activity of copper-containing oxidative systems affects the synthesis of biomass, the P_7 content of the fungal mycelium, and its amyolytic activity. Amyolytic activity rises, and the synthesis of energy-rich phosphates increases. As for biomass synthesis, diethyldithiocarba-

TABLE 6. Effect of Diethyldithiocarbamate (DDC) and Azide on the Respiration Rate of the Fungus, the Synthesis of Biomass, Amyolytic Activity, and Amount of P_7

Experiment	Experimental variant	Dry weight of mycelium, g	α -Amylase activity in		P_7 per dry material in		Respiration as O_2 consumed in 10 min, in mm ³	
			conditional units	% of control	mg %	% of control	per 1 g of dry mycelium	in % of control
I	Control	2.41	-	-	325	100.0	374.8	100.0
	0.0002 M NaN_3	1.94	-	-	306	91.1	37.5	10.0
	0.0002 M DDC	2.00	-	-	761	234.1	195.1	52.1
II	Control	2.58	1370	100.0	208	100.0	761.2	100.0
	0.0001 M DDC	2.34	1600	116.9	221	106.2	515.2	67.7
	0.0002 M DDC	2.38	1600	116.9	-	-	321.3	42.2
	0.0005 M DDC	No growth						

TABLE 7. Effect of Methylene Blue (MB) and 2,6-Dichlorophenolindophenol (DPI) on the Synthesis of Biomass, P_7 , Amyolytic Activity, and Respiration of the Fungus

Experi- ment	Experimental variant	Dry weight of myceli- um,g	Amylase activity				P ₇ per dry ma- terial in		Respiration as O ₂ consumed in 10 min, in mm ³	
			α-amylase		total					
			condition- al units	% of control	condition- al units	% of control	mg %	% of control	per 1 g of dry ma- terial	in % of control
I	Control	2,10	1371	100.0	11,1	100.0	193	100	376,8	100.0
	0,00001 M MB	2,16	1622	118.9	12,1	108,1	-	-	-	-
	0,00005 M MB	1,68	1371	100.0	11,1	100.0	313	162	218,7	58,1
	0,00005 M DPI	2,02	1471	107.3	11,6	104,5	280	145	84,4	22,4
II	Control	1,79	1040	100.0	7,5	100.0	-	-	-	-
	0,00001 M DPI	1,71	1830	175.9	9,4	126,7	-	-	-	-

mate in a concentration of 0.0001-0.0002 M reduced it somewhat, although this still does not suggest a low coefficient of utilization of the carbohydrates of the medium.

On the basis of the data presented, it can be noted that the partial inhibition of respiration in the organism brings about a rearrangement of its metabolism, leading to an increase in its content of energy-rich phosphates and an increase in the activity of some carbohydrases.

Experiments with Some Redox Indicators

The inhibition of respiration in *A. oryzae* by means of the partial uncoupling of the action of iron- or copper-containing oxidases can be linked with a shift in the oxidation-reduction potential of these systems, since the uncoupling of iron- or copper-containing oxidases is the result of blocking the active valences of the metal, which is also associated with the lowering of the oxidation potential. Experiments in which the effect of certain redox indicators on the respiration of the fungus, its amylolytic activity, biomass synthesis, and P_7 content were studied were of considerable theoretical interest. The experiments were set up with two concentrations of methylene blue and 2,6-dichlorophenolindophenol.

The data presented in Table 7 are evidence of the fact that low concentrations of the redox indicators methylene blue and 2,6-dichlorophenolindophenol promote the increase of amylolytic activity, an increase in the amount of energy-rich phosphates, and for methylene blue in a concentration of 0.00001 M—a higher rate of biosynthesis. Furthermore, both indicators had a considerable inhibitory effect on the respiration of the fungus.

We did not make a thorough study of the mechanism of action of redox indicators on the metabolic reactions of the fungus. It must be assumed that, in the presence of redox indicators, a shift occurs in the oxidation-reduction potential of the intracellular oxidation-reduction systems of the growing fungus. Such a conclusion can also be reached a priori, since the oxidation-reduction potential of such a strongly respiring organism as *A. oryzae* must be highly positive ($E_0 = +800$). According to data in the literature, on the other hand, the redox indicators employed are capable of producing a relatively low potential in medium at pH 7.0. Thus, methylene blue can produce an oxidation-reduction potential of $E_0 = +11$ mv, whereas 2,6-dichlorophenolindophenol can produce an oxidation-reduction potential of $E_0 = +217$ mv.

SUMMARY

1. Sodium fluoride in a concentration of 0.001-0.0025 M promotes the synthesis of fungal biomass, the increase of its P_7 content, an increase in α -amylase activity, and causes partial inhibition of respiration.

2. Sodium azide causes the inhibition of respiration and reproduction of the fungus, a reduction in its P_7 content and, at low concentrations, increases amylolytic activity.

3. When 5 mg % of Fe^{++} is added to the nutrient medium, the negative effect of sodium azide on P_7 formation is reduced, and the amylolytic activity of the fungus increases.

4. Diethyldithiocarbamate reduces the absolute yield of mycelium slightly, inhibits fungal respiration, and promotes an increase in the P_7 content and an increase in amylolytic activity.

5. Methylene blue in a concentration of 0.00005 M partially inhibits fungal respiration, promotes the formation of P_7 in it, and somewhat reduces the growth rate of the mycelium. A concentration of 0.00001 M activates the synthesis of biomass and the production of amylolytic enzymes by the fungus.

6. 2,6-Dichlorophenolindophenol at the same concentrations inhibits fungal respiration more strongly than methylene blue, reduces the synthesis of biomass somewhat, and promotes an increase in the P_7 content and an increase in amylolytic activity.

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ADAPTATION OF *Nitrosomonas* TO EXISTENCE CONDITIONS ON VARIOUS NATURAL SUBSTRATES

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A study of the ecological characteristics of various *Nitrosomonas* strains has shown that upon certain natural substrates there develop ecological types of nitrite bacteria adapted to living under the given conditions.

We investigated pure and cumulative *Nitrosomonas* cultures in their relationship to the active reaction of the medium, to ammonium salts of inorganic and organic acids, and to extracts of the substrates from which the cultures were isolated.

The optimal hydrogen ion concentration for *Nitrosomonas* growth in mineral media lies, as is known, in the alkaline zone — the pH, however, has not been definitely determined. This, in our opinion, is due to the fact that the various investigators have dealt with various strains of nitrite bacteria isolated from differing ecological habitat conditions, and therefore varying in their optimal pH requirements (Meyerhoff, 1916, 1917; Gaarder and Hagem, 1922; Meek and Lipmann, 1922; Nelson, 1931; Winogradsky, 1952; Bömeke, 1939; Hofman and Lees, 1952, 1953; da Silva, 1952; Buswell, et al., 1954).

METHODS

Our experiments dealing with the influence of hydrogen ion concentration on the activity of cumulative and pure *Nitrosomonas* strains isolated from various natural substrates were conducted in buffer mixtures to which Winogradsky medium was added. We used Sørensen's buffer mixture, consisting of Na_2HPO_4 and KH_2PO_4 . Winogradsky's medium was used in double its usual concentration, taking into account 50 ml of medium to 100 ml of buffer mixture. The nitrifying activity of the cultures was tested at 16 different pH values, from 4.94 to 9.18. In the course of the experiments a repeat pH measurement was made by the electrometric method and by the color scale method using universal Merck indicator (pH 4 - 9). To sustain the reaction at a certain level, we used diluted hydrochloric acid (2-5%) and a solution of sodium carbonate (0.5M). The Winogradsky medium (pH 7.18 - 7.21) served as the control.

The reaction of the medium as an influence on the culture's life processes was evaluated by the intensity of

nitrification, measured by conducting a quantitative determination of the nitrites in the medium (Ul'yanova, 1961).

RESULTS

A study of the influence of the hydrogen ion concentration on the activity of cumulative and pure *Nitrosomonas* cultures obtained from various natural media showed that the majority of cultures were adapted to the hydrogen ion concentrations occurring in their habitats (Table 1 and figure).

Consequently, the acidity of the earth, as such, does not always hinder the nitrification process.

This has been noted by a number of authors, Griffith and Manning (1949) and Kaufmann and Boquel (1953) discovered an active nitrification process by *Nitrosomonas* strains inhabiting various soils of Africa (jungle soil and Uganda soil with pH 5.0-5.5). A similar phenomenon was observed by Paul and Shariff (1954) in their study of the nitrification process in siliceous soils of British Guiana (pH 4.5).

In a number of nitrite bacteria cultures which we isolated from sandy soils, alkaline soils, and grassy black earth in forested belts (the Kamen Steppe), the optimal pH differed from the pH of the natural substrates. These bacteria developed better in an alkaline medium reaction than in the neutral or acid one characteristic of the media from which the cultures were obtained. This shows that the optimal pH value in the laboratory culture does not always coincide with what is observed in natural conditions of *Nitrosomonas* habitation. Heubült (1929), in working with *Nitrosomonas* isolated from soil of pH 5.6-6.8, likewise found that the organism, in a fluid inorganic medium, developed better at a pH of 7.1-7.2.

In our comparative study of cumulative and pure *Nitrosomonas* cultures isolated from various natural media, we observed likewise the rate of ammonia oxidation from the ammonium salts of inorganic acids (sulfuric, orthophosphoric, nitric) and organic (malic) acid.

Solutions of the above-mentioned ammonium salts were sterilized separately and added in varying amounts to Winogradsky medium, which also served as the con-

trol. The ammonium salts were used in concentrations of 0.01 to 6M. The pH varied from 7.19 to 7.25. Speed of ammonia oxidation from ammonium salts of the various acids was evaluated by the time required for the appearance of a positive nitrite reaction, and by the quantity of nitrites formed. The experiments were stopped after 22 days of cultivation.

The work conducted demonstrated that the best development of the investigated cumulative and pure nitrite bacteria cultures occurred upon the addition to the Winogradsky medium of various ammonium salts in small amounts — from 0.01 to 0.4 M. In most cases the pure and cumulative cultures formed their maximum quantities of nitrites upon addition of 0.01 M of each of the ammonium salts to the Winogradsky medium.

As compared to the Winogradsky medium (control), of the ammonium salts tested those exerting the most favorable influence on raising culture activity were the phosphate, sulfate and malate (this latter was especially favorable for cumulative cultures).

Ammonium nitrate considerably depressed the nitrification process in the tested cultures. This may be connected with the negative influence of the NO_3^- ion (Table 2).

Consequently, the anions of the various ammonium salts likewise exert a significant influence on nitrite formation by various *Nitrosomonas* strains. We must note that the cumulative and pure *Nitrosomonas* strains isolated from substrates with a higher organic matter content (active sludge, various black earths, soda sulfate alkaline earths) are characterized by a higher rate of ammonia oxidation from the ammonium salts of the

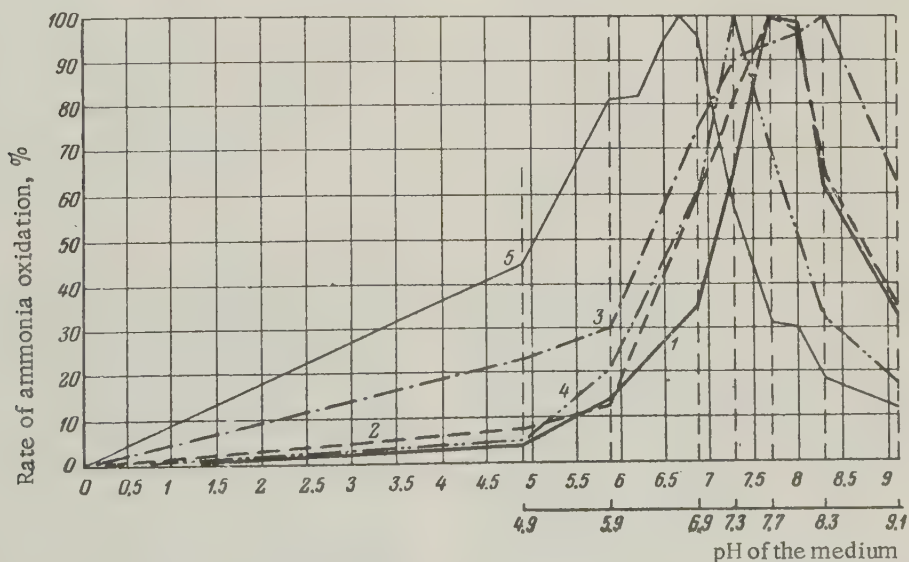
inorganic and organic acids studied. The chemical characteristics of the natural substrates from which the cultures were obtained, and the organic matter content of these substrates, were presented in our first communication (Ul'yanova, 1960).

Nitrosomonas strains isolated from substrates of low organic content (e.g., sandy soils) significantly lag behind the above-mentioned strains in their oxidation rate of the ammonium salts indicated above. The slowest oxidation rate of the various ammonium salts was exhibited by the *Nitrosomonas* strains found in the high mountain soils of Bulgaria. These latter cultures (from the sandy and mountainous earths) proved, likewise, more sensitive to increased concentrations of the various ammonium salts in the medium, and stopped developing at markedly lower salt concentrations than the cultures from the active sludge, black earths, and soda sulfate alkaline earths.

This varying oxidation ability in *Nitrosomonas* strains can, apparently, be explained as follows.

Media containing high amounts of organic matter abound in active forms of ammonifiers which carry on an intensive process of mineralization. In such media the *Nitrosomonas*, obtaining sufficient amounts of ammonia, possess a great ability for its oxidation. Examining this material, we find a complete analogy with data on the nitrification activity of the nitrite bacteria strains from the various habitation media studied (Ul'yanova, 1961).

These facts likewise emphasize the dependence of *Nitrosomonas*' physiology on the ecological nature of the substrate in which it is growing.



Influence of reaction of medium on the activity of pure *Nitrosomonas* cultures isolated from various natural substrates. 1) Culture from active sludge; 2) from Bulgarian black earth; 3) from alkaline earth of the Kamen Steppe; 4) from sandy soil of Southern Karelia; 5) from mountainous earth of Bulgaria.

TABLE 1. Optimal pH's for Cumulative and Pure Cultures of Nitrite Bacteria Isolated from Various Natural Substrates

Substrates from which the cumulative and pure nitrite bacteria cultures were isolated	pH of the natural substrates	Cumulative cultures	Pure cultures
		optimal pH	optimal pH
Active sludge from waste water reservoirs	7.87	8.34	7.73
Horse manure	7.63	8.04	7.73
Cow manure	7.84	8.04	8.04
Black earth of the Kamen Steppe	6.28	8.34	8.04
Black earth of Bulgaria	7.80	7.73	7.78
Black earth from the Tellerman Experimental Forestry	7.26	7.73	7.38
Alkaline earth of the Kamen Steppe	7.70	8.04	8.34
Alkaline earth from the Tellerman Experimental Forestry	7.50	8.04	8.04
Dark grey forest earth from the Tellerman Experimental Forestry	6.99	7.38	7.17
Sandy soil from Southern Karelia	5.13	7.38	7.38
Sandy soil from the Tellerman Experimental Forestry	5.55	7.73	7.73
Mountainous earth from Bulgaria	5.80	6.98	6.64

The influence of extracts of various natural media on the nitrification activity of pure *Nitrosomonas* cultures likewise speaks for the adaptability of the organism studied to the specific conditions of its habitat. The preparation of media with extracts of various substrates was described earlier (Ul'yanova, 1960).

As control we used Winogradsky medium, to which we added the same amount of $(\text{NH}_4)_2\text{SO}_4$ as to the extracts (i.e., 0.1%). The dry residue content of the extracts varied between 0.1% and 0.9%. The experiments were conducted in duplicate and continued for two weeks. The influence of the extracts on growth and development of the pure cultures was judged by the speed of appearance of a positive nitrite reaction, by the quantity of nitrites formed and by a cell count (this latter by a direct microscopic count; in each case 50 visual fields were counted and average figures were taken).

The results showed that of the pure *Nitrosomonas* cultures studied, the majority accumulated more cells and formed somewhat larger amounts of nitrites when grown on extracts from their habitats than when developing on the Winogradsky medium (control). The numerical data are presented in Table 3.

All other extracts, except those corresponding to the substrate from which the *Nitrosomonas* cultures were obtained, in most cases exerted no noticeable influence on culture activity. This phenomenon is really noteworthy, since the ecology of *Nitrosomonas* should be studied from every aspect. In the process of adaptation to ecological conditions the various *Nitrosomas* strains must

have developed certain adaptations to the specific conditions of one natural medium or another. That is why we consider it necessary to grow the tested cultures on extracts of the substrates from which they were isolated. It is quite clear that in the study of microorganisms one should not overlook the natural composition of nutrient media and the microbes' relationship to them. In order to accurately determine the quantitative composition and physiological traits of various microorganisms, it is essential to employ specific nutrient media prepared from extracts of the natural substrates being investigated.

Many authors have repeatedly noted in the literature that the mineral nutrient medium composition for cultivating *Nitrosomonas* in laboratory conditions does not provide for its maximum growth, since the medium is incomplete. This circumstance has stimulated scientists' efforts to approximate the media to the natural habitat conditions of the nitrite bacteria. This has resulted in the use of extracts from various soils, from dry leaves, manure, humic acids and their salts, and other substances. We find, however, contradictory data in the literature concerning the influence of these substances on the growth and development of *Nitrosomonas*.

A number of authors note a favorable influence of extracts from different soils, decayed leaves, peat humates and humic acid salts on the nitrification process (Karpinski and Niklewski, 1897; Winogradsky and Omelyanskii, 1899; Maksinoff, 1909; Gibbs, 1919; Imshenetskii and Ruban, 1952). On the other hand, Nelson (1931) Imshenetskii and Ruban (1953) and Meiklejohn (1953),

TABLE 2. Nitrite Formation by Cumulative and Pure Nitrosomonas Cultures on Wino-gradsky Medium and on the Same Medium with added Ammonium Salts of Inorganic and Organic Acids (NO₂ mg/liter in 22 days)

Substrates from which the cumulative and pure cultures were isolated	Cumulative cultures					Pure cultures				
	(NH ₄) ₂ SO ₄ 0.01 M	(NH ₄) ₂ HPO ₄ 0.01 M	NH ₄ NO ₃ 0.01 M	NH ₄ malate, 0.01 M	Wino-gradsky medium	(NH ₄) ₂ SO ₄ 0.01 M	(NH ₄) ₂ HPO ₄ 0.01 M	NH ₄ NO ₃ 0.01 M	NH ₄ malate, 0.01 M	Wino-gradsky medium
Active sludge from waste water reservoirs	4400	4800.0	2880	4870.0	1165.0	29.9	26.10	11.30	27.80	19.24
Kamen steppe black soil	1820	2742.0	910	4500.0	763.2	25.7	23.90	9.70	26.10	18.35
Bulgarian black soil	1400	1972.7	2010	4154.0	635.4	26.6	28.30	10.70	24.30	17.11
Tellerman experimental forestry black soil	575	1874.3	500	3420.0	515.5	20.9	19.10	8.90	17.40	16.31
Kamen steppe alkaline soil	3850	1711.6	820	3880.0	405.7	29.1	18.50	5.10	20.50	14.96
Southern Karelia sandy soil	425	479.0	309	967.0	309.8	14.9	15.80	15.01	16.14	13.18
Tellerman experimental forestry sandy soil	352	513.0	321	870.0	265.0	16.4	11.01	6.90	13.48	12.26
Bulgarian high mountain soil	327	343.1	303	359.6	243.9	10.7	11.60	5.50	14.25	8.14

TABLE 3. Nitrite Formation and Cell Quantities in Nitrite Bacteria Grown on Wino-gradsky Medium and on Extracts of Substrates from which the Cultures were Isolated, as Observed during a 14-Day Period

Substrates from which the pure cultures were isolated and the extracts prepared	NO ₂ , mg/liter, and no. of bacteria/ml medium			
	Wino-gradsky medium		extracts	
	nitrites	number of cells	nitrites	number of cells
Tellerman experimental forestry black soil	15.89	27.5·10 ⁵	16.05	31.0·10 ⁵
Kamen steppe black soil	17.01	54.3·10 ⁵	18.45	67.0·10 ⁵
Bulgarian black soil	16.44	39.6·10 ⁵	19.10	71.2·10 ⁵
Tellerman experimental forestry dark grey forest soil	15.13	24.5·10 ⁵	18.21	70.1·10 ⁵
Tellerman exp. forestry alkaline soil	13.21	20.4·10 ⁵	15.95	30.0·10 ⁵
Southern Karelia sandy soil	12.29	17.6·10 ⁵	14.87	21.5·10 ⁵
Bulgarian high mountain soil	7.89	9.8·10 ⁵	6.45	6.5·10 ⁵
Cow manure	16.85	34.8·10 ⁵	22.75	107·10 ⁵

working with extracts from soil and plant residues, noted their negative effect on the growth and ammonia oxidation of Nitrosomonas. Such contradictory data, obtained in studying the influence of similar substances on nitrification, bear witness to the insufficient knowledge of this phenomenon and demand further work on the subject.

We consider that this contradiction is only a seeming one. The above works lack an ecological approach in their study of Nitrosomonas. The authors do not indicate from what types of soil they isolated the nitrite bacteria cultures (they consider "soil in general"), and which extracts acted on their development (whether extracts of soils from which the cultures were isolated, or

others). Therefore it is difficult to account for the negative influence of extracts noted by a number of researchers. Moreover, it is impossible to do this because at the present time there exists no accurate chemical characterization of soils and their extracts. So it is not surprising that in factual material concerning specific traits of Nitrosomonas in pure culture we encounter unmatching and contradictory data.

Since we did not arrange for a chemical analysis of the extracts, it is hard to explain why extracts of soils from which the cultures were isolated exerted a stimulating effect on their development. Possibly this stimulation was due to some growth factors, or such an effect may be explained by the presence in the extracts of

certain microelements. These latter, as shown by Meiklejohn (1950), influence pure cultures of Nitrosomonas favorably.

Future studies on the Nitrosomonas should be directed to foster a more ecological approach to this subject.

The material we have presented in the above communication shows a definite ecologic adaptation of various Nitrosomonas strains to existence on varying natural substrates.

SUMMARY

1. The conditions of the habitat definitely affect the ecology and physiology of Nitrosomonas strains.

2. In most cases the optimal pH's of pure and cumulative Nitrosomonas cultures coincide, under laboratory conditions, with the pH of natural substrates from which they have been isolated.

3. The cumulative and pure cultures of nitrite bacteria isolated from various natural substrates show a different rate of oxidation of various ammonium salts. A high oxidation rate is appropriate to cultures from substrates abundant in organic matter. Much inferior in the rate of oxidation of ammonium salts are cultures from substrates with a lesser content of organic matter.

The anions of the ammonium salts tested likewise appreciably influence the nitrification process in various Nitrosomonas strains.

4. Media prepared on extracts of substrates from which the Nitrosomonas strains have been isolated favor the formation of a greater number of cells and hence the accumulation of a somewhat larger amount of nitrites as compared with their culture on the Winogradsky medium (control).

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. Some or all of this periodical literature may well be available in English translation. A complete list of the cover-to-cover English translations appears at the back of this issue.

ORIGIN OF CERTAIN ANTIBIOTICS UNDER NATURAL CONDITIONS

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As the result of numerous investigations, many new antibiotics have been described in the last ten years. But during searches for antibiotics, due to the endeavor to find new ones as rapidly as possible, little attention is devoted to their ecological significance. In the majority of cases, only the number of strains isolated and the presence of antibiotic activity in them are given in the work, without any attempt to explore more thoroughly their behavior in the extremely complex competitive interrelationships of the soil microflora.

The value of the direct determination of antibiotic production under natural conditions in the soil is frequently overestimated and is regarded as necessary to judge the ecological meaningfulness of the antibiotics. The original difficulty in this rather one-sided approach to the demonstration of the production of antibiotics in sterile unenriched medium (Ehrlich, Anderson et al., 1952; Evans and Gottlieb, 1955; Gottlieb and Siminoff, 1952; Gottlieb, Siminoff and Martin, 1952; Krasil'nikov, 1954; Martin and Gottlieb, 1952; Siminoff and Gottlieb, 1951) and in unenriched medium with normal microflora (Gregory, Allen et al., 1952; Hessayon, 1951, 1953; Wright, 1952) sometimes led to some hasty conclusions denying any ecological significance of antibiotics in the soil (Waksman, 1956).

The most recent works of Wright (1956a, b, c) showed that sources of nutrients in soil are rarely present in such composition and quantity as to ensure the growth of microorganisms and noticeable antibiotic production. This does not mean, however, that antibiotics can not serve as an effective means of microbial competition in the soil surrounding the microcolonies which are formed in the vicinity of vegetable particles. An exhaustive critical review on this subject can be found in Brian (1957). The possibility of the origination of certain types of antibiotics from the point of view of prolonged natural selection has been investigated only occasionally. Cooper and Chilton (1950) found that 18-31% of 7302 actinomycetes isolated from 181 soil samples from sugar cane plantations had activity against Pythium arrhenomanes, which sometimes causes root rot of sugar cane. Meredith (1946) isolated an actinomycete with antagonistic activity against Fusarium oxysporum var.

cubense from the soil of banana plantations. Schatz and Hazen (1948) isolated actinomycetes from ecologically different soil samples and showed that in forest soils there is a large number of actinomycetes which are active with respect to certain fungi pathogenic for humans. Wallhäuser (1951) showed that each of 23 bacteria and each of 16 fungi isolated from a single soil sample exhibited mutual antagonism to a considerable extent. Szabó (1956) studied the relationship among microorganisms growing on small soil particles, and came to the conclusion that as high as 43% of the interrelationships were of an antagonistic character.

The present work is devoted to the question of to what extent the antimicrobial spectrum of activity of antagonistic actinomycetes is connected with ecological conditions in various soils, inasmuch as these conditions can produce various competitive relationships among microorganisms, and to what extent it is possible to regard antibiotic-producing actinomycetes as ecologically significant in soil in this connection.

METHODS

Materials. The soil samples were collected in various places in the loess regions of China, from virgin forest and forest soils and from cultivated soils, both from the rhizosphere and outside of it. From 194 soil samples, 931 actinomycetes were isolated which were active with respect to antibiotics. Of this number, the nature of the antibiotics produced was tentatively determined in 832 (94.5%).

Isolation methods. The actinomycete strains were isolated from soil by the centrifugation method (Reháček, 1956). This method makes it possible to obtain colonies of pure cultures of actinomycetes which can be transferred directly from the first culture medium and used for further study. The strains isolated were grown in Petri dishes for 5-8 days. Then, agar blocks with the actinomycetes were cut out and used for determinations of antibiotic spectrum, solubility, ionic character, and absorption spectrum in uv of the antibiotic produced. Approximate determinations of the taxonomic position of the producer were made. For details of the methods, see Vanek et al. (1958).

During the course of the year, considerable fluctuations were observed in the number of actinomycetes in the soil as a function of external conditions such as temperature, moisture, etc.

In order to minimize the fluctuations in the results caused by these factors, we subdivided the strains isolated from each sample into groups according to the nature of the antibiotic produced and preliminary taxonomic identification. Regardless of the number of strains isolated from the given sample and producing identical antibiotics, only different antibiotics were used and regarded as different cases in the calculation.

RESULTS

From 62 samples of virgin forest and forest soils, 283 antibiotically active strains (118 cases) were subjected to analysis; from 61 samples of cultivated nonrhizosphere soil, 378 antibiotically active strains (110 cases) were analyzed; from 71 samples of cultivated rhizosphere soils, 219 antibiotically active strains (120 cases) were analyzed.

It is seen from Fig. 1 that, among the actinomycetes isolated from forest and virgin forest soils, 43% of cases of activity against Gram-positive bacteria (column A) were observed, 4% of cases of activity against Gram-

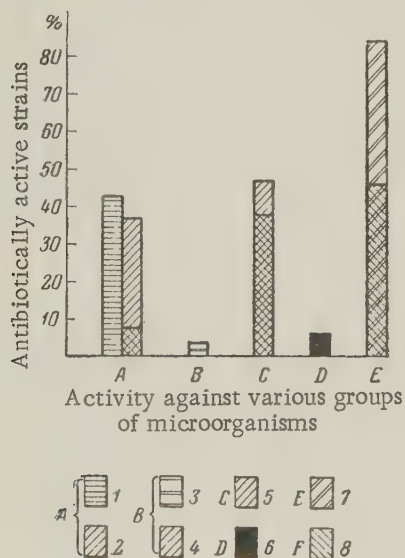


Fig. 1. Types of antibiotically active actinomycetes isolated from virgin forest and forest soils. A: 1) Gram-positive bacteria; 2) Gram-positive bacteria and fungi; B: 3) Gram-negative bacteria and Gram-positive bacteria; 4) Gram-negative and Gram-positive bacteria and fungi; C: 5) fungi only; D: 6) yeasts; E: 7) total number active against fungi (including fungi only and against fungi+bacteria); F: 8) antibiotics of a polyene nature. [Column F omitted in Figs. 1-3 in Russian original - Publisher.]

negative and Gram-positive bacteria (column B), 47% of cases of activity against fungi alone (column C), and 6% of cases of considerable activity against yeasts (column D). Of the 43% of cases of activity against Gram-positive bacteria, there was activity against Gram-positive bacteria and fungi in 37% of the cases, of which 29% of the cases were represented by antibiotics of a polyene nature, but in 6% of the cases there was activity against Gram-positive bacteria only. In 47% of the cases, the antibiotics were active only against fungi; in 10% of the cases they were represented by nonpolyene antibiotics, and in 37% of the cases by polyene antibiotics. According to column E, it can be seen that antifungal activity was observed in a total of 84% of the cases, 46% of which was due to antibiotics of a polyene nature.

Figure 2 presents the results of a study of antibiotically active actinomycetes isolated from cultivated nonrhizosphere soil. A considerable decrease in the relative number of cases of activity against fungi alone (3% column C) is seen as compared with the relative number of such cases (47%) among actinomycetes isolated from virgin forest and forest soils. On the other hand, in cultivated nonrhizosphere soil, the number of cases of activity against Gram-positive bacteria increased to 71% (column A), whereas the number of cases of activity against Gram-positive and Gram-negative bacteria increased to 25% (column B). Of the 71% of cases of activity against Gram-positive bacteria, there was activity against Gram-positive bacteria and fungi in 45% of the cases, including 35% of cases of nonpolyene and 10% of cases of polyene antibiotics. In 26% of the cases, activity was observed against Gram-positive bacteria only. Of the 25% of cases of activity against Gram-negative and Gram-positive bacteria, there was activity against Gram-negative and Gram-positive bacteria and fungi in 20% of the cases; this group of antibiotics was represented by polyenes. In 5% of the cases, there was activity against Gram-negative and Gram-positive bacteria only. According to column E, it can be seen that antifungal activity was noted in 67% of the cases, 12% of these being due to polyenes.

Figure 3 presents the results of the antibiotic activity of actinomycetes isolated from cultivated rhizosphere soil. A considerable increase in the relative number of cases of activity (48%) against Gram-negative and Gram-positive bacteria (column B) is observed here. On the other hand, the number of cases of antifungal activity alone decreased to 15% (column C) in comparison with 47% noted for actinomycetes isolated from forest or virgin forest soils. Of the 37% of cases of activity against Gram-positive bacteria (column A), there was activity against Gram-positive bacteria alone in 15% of the cases, but in 22% of the cases there was activity against Gram-positive bacteria and fungi. Of the latter number, there were nonpolyene antibiotics in 20% of the cases, and polyene antibiotics in 2% of the cases.

Of the 48% of cases of activity with respect to Gram-negative and Gram-positive bacteria, there was activity against Gram-negative and Gram-positive bacteria and fungi in 22% of the cases; polyene antibiotics were absent in this group. Activity against Gram-negative and Gram-positive bacteria alone was noted in 26% of the cases. Of the 15% of the cases of activity against fungi alone, 1% of the cases were represented by antibiotics of a polyene nature. From column E it can be seen that, of the total number of 6% of cases of antifungal activity, 3% of the cases were represented by antibiotics of a polyene nature.

DISCUSSION

A direct proof of the fact that strains which produce antibiotics can also produce them under natural conditions in the soil is regarded as decisive in judging their ecological significance. As the result of considerable efforts undertaken in this direction, it has been established that many microorganisms are capable of producing antibiotics even in unsterile and unenriched soil (Wright, 1952; 1956a, c). Many works have shown that microorganisms grow in the form of microcolonies in the soil, and that it is in just such a microenvironment that the production of antibiotics occurs. In those cases where is no proof of the production of an antibiotic in the soil, as in the case of *Streptomyces venezuelae* for example, it can be assumed that suitable conditions have not been found for obtaining significant growth of the actinomycete, and consequently, conditions for the production of antibiotics as well. According to the data of Ehrlich et al. (1953), the number of colonies in natural soil inoculated with *S. venezuelae* decreased from 10^9 to $4 \cdot 10^8$ colonies per 1 g of dry soil after three weeks. It is not surprising that it is impossible to demonstrate the production of chloramphenicol under such conditions.

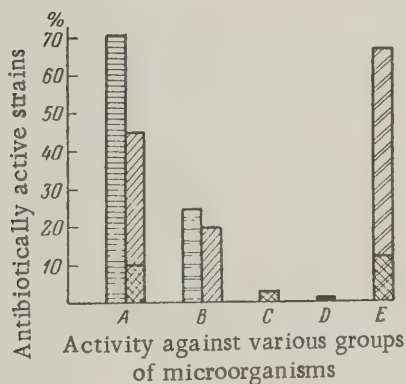


Fig. 2. Types of antibiotically active actinomycetes isolated from cultivated nonrhizosphere soil. The designations in Figs. 2 and 3 are the same as in Fig. 1.

Supposedly, one can obtain a clearer picture of the ecological significance of antibiotics in the soil by means of an analysis of the ecological conditions under which the microorganisms have grown in the soil over a long period of time. If the ability to produce an antibiotic of a certain type is a characteristic of the actinomycete which has developed and was fixed during the course of its historical development, the classification of competitive relationships in ecologically different types of soil is not determined by the seasonal changes in the number of microorganisms in the soil, which are influenced by temperature, moisture, uncontrolled supply of nutrients, etc., but is rather determined by the relative number of the actinomycetes producing certain types of antibiotics which are active against the microflora characteristic for the given soil sample.

In the present work, where we proceeded on the basis of the hypothesis mentioned above, it was found that, in the collection of antibiotically active actinomycetes isolated from ecologically different soil samples, different relative numbers of producers with particular types of spectra of antibiotic activity are noted. A significant increase in the relative number of actinomycetes with an antifungal spectrum in forest and virgin forest soils can be connected with a relatively increased number of fungi in these types of soils (Jagnow, 1956). On the other hand, an increase in the number of actinomycetes active with respect to Gram-positive bacteria in cultivated nonrhizosphere soils can be connected with the presence of an increased number of Gram-positive bacteria in these soils, where their number rises 20- to 30-fold through the effect of cultivation (Burgess, 1958). An increased number of actinomycetes active against Gram-negative bacteria in rhizosphere soils can be connected with a relatively increased number of Gram-negative bacteria in these rapidly forming sectors of the soil (Starkey, 1958). However, the interrelationships in the rhizosphere are extremely complex. According to the data of Krasil'nikov (1954) and of Rouatt et al. (1951),

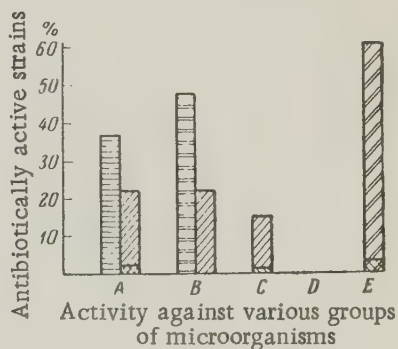


Fig. 3. Types of antibiotically active actinomycetes isolated from cultivated rhizosphere soil.

it was found that the total number of antibiotic-producing actinomycetes which can be isolated from the rhizosphere is less than that which can be isolated from non-rhizosphere soil. The greater supply of nutrients associated with an increased number of bacteria and intensive metabolism is not manifested by an increase in the total number of antibiotically active actinomycetes. It can therefore be assumed that, in this case, other effective forms of antagonism must exist (Brian, 1958), such as the capacity for more rapid growth and utilization of nutrients in bacteria for example.

SUMMARY

1. Actinomycetes which produce antibiotics with antifungal activity alone or with antibacterial activity as well were found in 90% of the cases in virgin forest and forest soils, in 67% of the cases in cultivated nonrhizosphere soil, and in 61% of the cases in cultivated rhizosphere soil. Of the total number of cases of antifungal activity, antibiotics of a polyene nature constituted 46% of the cases in actinomycetes isolated from virgin forest and forest soils, 12% of the cases of actinomycetes isolated from cultivated nonrhizosphere soil, and 3% of the cases of actinomycetes isolated from cultivated rhizosphere soil.

2. Actinomycetes producing antibiotics active against Gram-positive bacteria were found in 71% of the cases in cultivated nonrhizosphere soil, in 43% of the cases in virgin forest and forest soils, and in 37% of the cases in cultivated rhizosphere soil.

3. Actinomycetes producing antibiotics active against Gram-negative and Gram-positive bacteria were found in 48% of the cases in cultivated rhizosphere soil, in 25% of the cases in cultivated nonrhizosphere soil, and in 4% of the cases in forest and virgin forest soils.

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LYTIC CAPACITY OF ACTINOMYCETES OF THE ORANGE GROUP

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It is well known that many species of actinomycetes are capable of lysing cells of Gram-positive and Gram-negative bacteria.

The lysis of bacteria by actinomycetes was first described by Lieske (1921), then by Gratia and Dath (1924, 1925a, b, 1926), Welsch (1941, 1942, 1947), McGarty (1952), and Pakuta et al. (1954).

In 1957, Carter and others observed the phenomenon of lysis of a fungus (*Glomerella cingulata*) under the influence of the antibiotic actidione and actinomycete No. 55. Unfortunately, however, we do not know to what species culture No. 55 belongs. The culture of actinomycete No. 55 lysed live and dead mycelium, while the antibiotic lysed only live mycelium.

We studied the phenomenon of cell lysis of bacteria and fungi by cultures of orange actinomycetes from our collection. The results of these investigations are presented in this paper.

METHODS

The ability to lyse bacteria and fungi was checked in 62 cultures of orange actinomycetes.

The following test cultures were used to study the lytic capacity of the actinomycetes. Among the bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Serratia marcescens* (Bact. prodigiosum), *Pseudomonas pyocyanea*, *Bacillus subtilis*, *Bacillus mycoides*, *Mycobacterium B-5*, *Mycobacterium citreum*. Of the yeasts: *Saccharomyces cerevisiae* and *Candida albicans*. Of the fungi: *Fusarium oxysporum*, *Fusarium gramineum*, *Helminthosporium sativum* and *Alternaria solani*.

Two methods were used to carry out the investigation. First, cultures of orange actinomycetes were grown on various agar media for 7-9 days at 27°C. Then, blocks were cut out from them which were placed on the surface of test cultures grown for 1, 2, and 3 days on different media in Petri dishes—namely: bacteria on meat-peptone agar, fungi on potato agar, and yeasts on wort agar. The Petri dishes with the blocks were placed in an incubator at 27°C for 1-2 days. The results were determined by the production of zones of lysis of the test cultures around the blocks.

Second, paper discs (5 mm in diameter) were saturated with native culture fluids of the actinomycetes. For this purpose, the actinomycetes were grown in fish medium on a shaker for 7-9 days. At the same time, we also used paper discs saturated with the alcoholic extract of the antibiotic. The discs were placed on the surface of previously grown test cultures (1-, 2-, and 3-day -old). The results were recorded following 24-48 hr. The lytic capacity of the actinomycetes tested was determined by the size of the zone of absence of growth around the discs.

In the present work, actinomycetes of the orange group, which were described by us earlier, were tested. The actinomycetes of this group were subdivided into six subgroups on the basis of morphological characteristics (Yuan' Tsz-Shén, 1961).

RESULTS

The results of the investigations are presented in Table 1. As seen from the table, the lytic capacity exhibited by the different species of orange actinomycetes was not the same. The cultures of subgroups I, II and III had a stronger lytic capacity than the cultures of subgroups IV, while the cultures of subgroups V and VI had no lytic capacity. The lytic capacity of the orange actinomycetes was exhibited differently with respect to different test cultures: in *B. subtilis*, *B. mycoides*, *Mycobacterium B-5*, *M. citreum*, *F. oxysporum*, *F. gramineum*, *H. sativum*, and *A. solani* grown on agar for 1-2 days, the radius of the zone was 1-5 mm. At the age of three days, these same test cultures were not lysed.

The Gram-negative bacteria — *E. coli*, *S. marcescens*, and *P. pyocyanea*—and the yeasts *S. cerevisiae* and *C. albicans* proved to be resistant to the lytic action of the orange actinomycetes.

The microscopic picture of cell lysis is presented in Figs. 1a, 1b, 1c, 2a, 2b, 3a, and 3b. After the blocks of actinomycete cultures were placed on the surfaces of 1-2-day backgrounds of fungi or bacteria (Figs. 1a, 2a, 3a), a zone of absence of growth visible to the naked eye appeared around the block as early as after 5 hr; this zone increased to 3-5 mm in 1-1.5 days. In this

TABLE 1. Lytic Capacity of Orange Actinomycetes (agar blocks of cultures were tested)

Test cultures	S. aureus			E. coli			Serratia marcescens			P. pyocyanea			B. subtilis			B. mycoides			Mycobact. B-5			M. citreum			S. cerevisiae			C. albicans			F. oxysporum			F. gramineae			H. sativum			A. solani																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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Note: The figures show the radius of the zone of lysis of the test culture in mm.

zone, all of the fungal mycelium broke down almost completely with only fragments of hyphae and breakdown products remaining (Fig. 2b). Ghosts of lysed mycelium were encountered at the edge of the zone (Figs. 1b, 2b). It should be noted that the spores of *Fusarium* did not break down. An analogous picture was observed during the lysis of bacteria. After the blocks of actinomycetes or the paper discs saturated with antibiotics were put on, nearly all 24-hr-old bacterial cells broke down (Fig. 3b).

We also checked the viability of the lysed fungi and bacteria. For this purpose, small pieces from the zone of lysis were transferred to test tubes containing dilute wort or meat-peptone broth and were also inoculated on agar media using five test tubes for each test culture. It was found that the fungi *H. sativum* and *A. solani* and the bacteria *B. subtilis* and *Mycobacterium* B-5, lysed at the age of 24 hr, did not germinate.

Consequently, the lysed cells of fungi and bacteria did not retain viable forms.

In order to determine the conditions for the production of the lytic factor, the most active cultures of the orange actinomycetes of the first and third subgroups and the fungi sensitive to them — *H. sativum* and *A. solani* — were used. The actinomycetes were grown on various nutrient media:

Medium CP 1 (glucose—20 g, $MgSO_4$ —0.5g, $NaCl$ —0.5 g, KNO_3 —1 g, K_2HPO_4 —0.5 g, chalk—1 g, $FeSO_4$ —0.001 g, agar—17 g, water—1 liter, pH 7);

Fish medium (fish extract—10 ml, glucose—20 g, chalk—3 g, agar—17 g, water—1 liter, pH 7);

Corn medium (glucose—10 g, peptone—5 g, corn extract—5 g, $NaCl$ —5 g, chalk—5 g, agar—17 g, water—1 liter, pH 7);

Soy medium (soy bean meal—20 g, glucose—10 g, $NaCl$ —5 g, chalk—3 g, agar—17 g, water—1 liter, pH 7); potato and meat-peptone media. The results of this experiment are given in Table 2.

As seen from the data in this table, cultures of orange actinomycetes grown in fish medium exhibited the greatest lytic activity against fungi (the zone of lysis was 1-5 mm). Fish medium was apparently more favorable than corn, soy bean, potato, synthetic, and meat-peptone media for the production of lytic substances by the actinomycetes.

In order to clarify the question of the nature of the lytic substance of the actinomycete, cultures of orange actinomycetes were grown in fish medium for 7-9 days and then the lytic capacity of the culture fluid was studied according to the following scheme:

- Culture fluid (control);
- Culture fluid filtered through a Seitz filter;
- Culture fluid passed through activated carbon;
- Culture fluid boiled at 100 C for 20-30 min in a water bath;

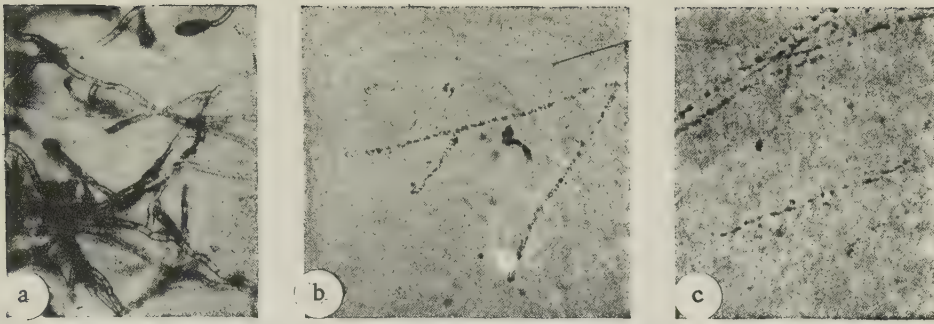


Fig. 1. Helminthosporum sativum. a) At the age of 2 days (control); b) following lysis. [Key c does not appear in original Russian caption—Publisher.]

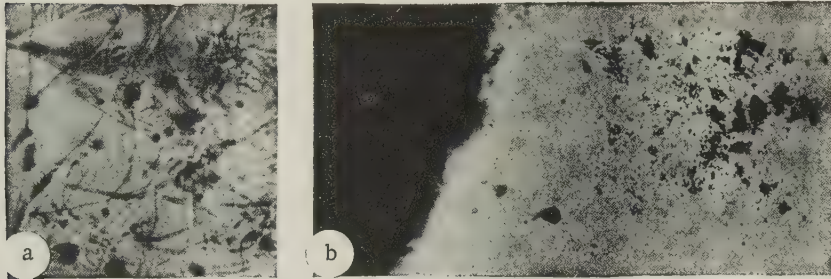


Fig. 2. Fusarium graminearum. a) At the age of 1-2 days (control); b) lysed culture from area near actinomycete block.

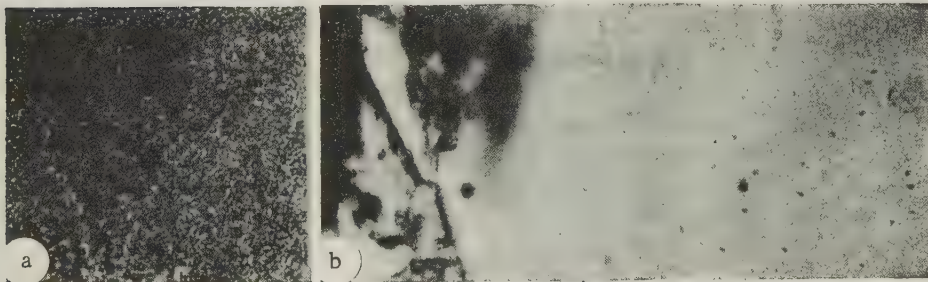


Fig. 3. B. subtilis. a) At the age of 1 day (control); b) following lysis around actinomycete block.

TABLE 2. Effect of the Composition of Nutrient Media on the Lytic Capacity of Actinomycetes

Cultures	Medium	H. sativum			A. solani		
		Age in days					
		1	2	3	1	2	3
Subgroup I	Fish	5	3	0	5	2	0
	Corn	4	3	0	3	1	0
	Potato	3	0	0	3	0.5	0
	Soy bean	3	3	0	3	1	0
	Meat-peptone	2	2	0	2	0	0
	CP I	3	3	0	3	1	0
Subgroup III	Fish	4	2	0	4	4	0
	Corn	3	2	0	3	3	0
	Potato	3	2	0	2	2	0
	Soy bean	2	1	0	2	2	0
	Meat-peptone	1	2	0	2	1	0
	CP I	2	2	0	2	2	0

Note: The designations in Tables 2-4 are the same as in Table 1.

TABLE 3. Lytic Activity of the Culture Fluid of Orange Actinomycetes Subjected to Various Treatments

Cultures	Scheme of experiment	H. sativum at age in days			A. solani at age in days		
		1	2	3	1	2	3
Subgroup I	Control (culture fluid)	4	2	0.5-1	3	1	0
	Culture fluid filtered through a Seitz filter	0	0	0	0	0	0
	Culture fluid passed through carbon	2	2	0.5	0.5	0	0
	Culture fluid boiled at 100° C for 20-30 min	3	2	0.5	3	2	0
	Culture fluid sterilized at 120 C for 20 min	3	1	0.5	2	1	0
	Culture fluid acidified to pH 2-3.2	2	1	0	2	1	0
	Culture fluid alkalized to pH 8-9	2	1	0	0.5	0.5	0
Subgroup II	Control (culture fluid)	3	2	0	2	1	0
	Culture fluid filtered through a Seitz filter	0	0	0	0	0	0
	Culture fluid passed through carbon	2	1	0	2	1	0
	Culture fluid boiled at 100 C for 20-30 min	3	2	0	1	0.5	0
	Culture fluid sterilized at 120 C for 20 min	3	1	0	2	0.5	0
	Culture fluid acidified to pH 2-3	3	1	0	2	0.5	0
	Culture fluid alkalized to pH 7-9	3	1	0	2	1	0

TABLE 4. Lytic Activity of Antibiotic 2886 from Culture No. 2886 of Subgroup I

Concen- tration, μg/ml	S. aureus			E. coli			Serratia marcescens			P. pyocyanea			B. subtilis			Mycobac- terium B-5			S. cerevisiae			C. albicans			H. sativum			A. solani			F. oxysporum					
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
Age in days																																				
50 5.5 1.8 0.6 0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			

e) Culture fluid sterilized in the autoclave at 120 C for 20 min;

f) Culture fluid acidified to pH 2-3;

g) Culture fluid alkalized to pH 8-9.

The results of this experiment are given in Table 3. It is seen from the data in Table 3 that only the culture fluid filtered through a Seitz filter did not exhibit lytic activity against fungi. It is important to note that the culture fluid boiled at 100 deg for 20-30 minutes, as well as the fluid sterilized at 120 deg for 20 minutes, had the capacity to lyse fungal mycelium. Consequently, the lytic activity is caused neither by actinophages nor by the culture itself, but apparently by antibiotic substances. We studied the lytically active substances obtained from a culture of actinomycete No. 2886 of the first subgroup*. The results of testing the effect of the antibiotic from culture No. 2886 on fungi and bacteria are given in Table 4. It is seen from this table that paper discs saturated with preparation No. 2886 lysed *B. subtilis*, *Mycobacterium* B-5, *H. sativum*, *A. solani*, and *F. oxysporum*. Lysis was observed only at a concentration of 1.8 μg/ml or higher; there was no lysis at lower concentrations.

In conclusion, the author offers his deep gratitude to N. A. Krasil'nikov and A. I. Korenyako, under whose direction the present work was carried out.

SUMMARY

1. It was found that orange actinomycetes are able to lyse cells of bacteria and fungi.
2. The lytic activity of various species of orange actinomycetes is different. It is more pronounced in cultures of subgroups I, II, and III than in IV. Cultures of subgroups V and VI have no lytic capacity.
3. Young 1-2-day cultures of fungi and bacteria are more susceptible to the lytic action of actinomycetes.
4. *Helminthosporium sativum*, *Alternaria solani*, *Fusarium gramineis*, *Bacillus mycoides* and *Mycobacterium* B-5 are more susceptible to the lytic action of actinomycetes than *Fusarium oxysporum*, *Mycobacterium citreum* or *Staphylococcus aureus*.
5. No lytic action of actinomycetes was noted with regard to Gram-negative bacteria, such as *Escherichia coli* (*Bacterium coli*), *Serratia marcescens* (*Bacterium prodigiosum*), *Pseudomonas pyocyanea* or with regard to yeasts, such as *Sacchromyces cerevisiae* and *Candida albicans*.
6. Of the media tested fish medium proved to be the most favorable for the production of lytic substances by orange actinomycetes.
7. The lytic factor of orange actinomycetes is a special substance of the antibiotic type.

* Purification was carried out by Yu. M. Khokhlova, for which the author offers her his appreciation.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

COMPARATIVE STUDY OF SOME BACTERIOPHAGES OF *Bacillus megaterium*

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As a rule, lysogenic cultures of bacteria and actinomycetes are resistant to internal temperate (symbiotic) phage. But, occasional observations are made when, as a result of mutation, temperate phage particles become genetically virulent and acquire ability to lyse the host.

It is known that lysogeny is somewhat widely distributed among most microorganisms. Many lysogenic cultures are observed in the bacilli, including also *B. megaterium* (Lwoff, 1935; Nagy, 1959; and others).

Certain strains of *B. megaterium* are utilized for production of phosphobacterin, a substance used as bacterial enrichment. The phenomenon of bacteriolysis is frequently observed in its production in factories manufacturing bacterial products. It has been shown by studies of Rautenshtein, Misюреva, Krongauz and Filatova (1960) that bacteriophages produced during lysis of *B. megaterium* under industrial conditions may differ from each other morphologically, in lytic and in other properties. There is a difference not only between phages produced during lysis of various industrial cultures, but also among phages produced during lysis of a single culture. The above-mentioned investigators established that in phage produced during lysis of a single industrial culture of *B. megaterium* some particles are capable and other are not able to lyse a given culture.

In this connection there arises a question pertaining to the origin of bacteriophages produced during lysis under industrial conditions and capable primarily to lyse the industrial culture.

In some cases, the cause of lysis pertains to external infection, i.e., the industrial culture is infected by phage externally during a stage of its multiplication upon interruption of sterility. In other situations, the culture itself may be the source of virulent phage, should it be lysogenic and some particles of internal symbiotic phage undergo genetic transformation and change from temperate to virulent phage.

The question of possible causes of lysis occurring in the course of utilization of *B. megaterium* var. phosphaticum strain No. 2 as industrial culture is considered in

the report by Rautenshtein et al. (1960). The culture was isolated and characterized by Menkina (1950). During lysis of this culture under industrial conditions the authors isolated two phages, designated as phage No. 16 and "Kiev" phage. The former is considered to be temperate phage of *B. megaterium* strain 2; the latter was virulent for this culture.

The present investigation was concerned with a comparative study of these two phages, with the purpose of clarifying their genetic relationship. Their serological properties were investigated, inasmuch as in the greater majority of cases genetically related phages are identical serologically. Certain other properties of these phages were also determined.

METHODS

The following cultures were used in studying bacteriophages: *B. megaterium* strain No. 2, its variant FChA (sensitive to both experimental phages, No. 16 and "Kiev"), as well as *B. megaterium* strains KM and mutilate. Phages of *B. megaterium* 899 W, M₁, M₅, Mii, Miii and 56.* Titration of experimental phages was carried out by overlay technique. The following were used as sources of nutrient media:

1. Peptone-corn agar composed of (in grams): glucose—10.0; NaCl—5.0; peptone—5.0; chalk—5.0; corn extract—5.0; agar—20.0; tap water—1,000 ml.

2. Yeast-casein agar of the following composition: acid casein hydrolysate in the amount of 150-160 mg % amino acid nitrogen; NaCl—2.5 g; KH₂PO₄—0.5 g; MgCl₂—0.4 g; soluble starch—1.5 g; CaCl₂—0.01 g; FeSO₄—0.01 g; CuSO₄—0.005 g; cysteine—0.03 g; yeast dialysate—50 ml; agar—25.0 g; tap water—1000 ml.

3. Hottinger's agar medium.

* The authors wish to express their appreciation to Prof. Ivanovich (Hungary) for kindly supplying these phages as well as cultures of *B. megaterium* strains KM and mutilate.

TABLE 1. Neutralization Reaction of "Kiev" and No. 16 Bacteriophages by Antisera Nos. 336 and 523 ("Kiev" phage) and No. 557 (phage No. 16)

Bacterio- phage	Antiserum No. 336				Antiserum No. 523				Antiserum No. 557				Normal serum of rabbit No. 504 (control)
	dilution				dilution				dilution				
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	
"Kiev "	Complete in- activation	Complete in- activation	Complete in- activation	Partial inac- tivation *	Complete inactivation				No inactivation				No inactivation
No. 16	No inactivation				No inactivation				Complete in- activation	Complete in- activation	Complete in- activation	Partial inac- tivation *	Same
No. 899	Same				Same				No inactivation				Same
No. 56	Not made				"				Same				"
Mii	Same				"				"				"
Miii	"				"				"				"
M ₁	"				"				Not made				"
M ₅	"				"				Same				"

* With partial inactivation of "Kiev" phage by antiserum No. 336 there were 93 plaques, with 535 plaques in the control; with partial neutralization of phage No. 16 by antiserum No. 557 there were 8 plaques, with complete lysis in the control.

Antiphage sera were obtained from rabbits immunized independently with phages No. 16 and "Kiev." The initial injection was made with Freund's adjuvants—lanolin, paraffin oil, and dried cultures of BCG. The following 13 injections were carried out at weekly intervals without adjuvants. The animals were bled nine days after the last, i.e., 13th, injection. Two antisera (Nos. 336 and 523) were collected from animals immunized to "Kiev" phage, and one (No. 557) to phage No. 16.

RESULTS

Serological properties of phages were determined by means of a neutralization test. Several experiments were carried out using the complement fixation technique. Results of the neutralization test are presented in Table 1.

As can be seen from Table 1, phages 16 and "Kiev" clearly differ from each other by their serological properties, and according to neutralization data do not contain common antigens.

Neutralization reaction of antiphage sera to these 2 phages with phages of B. megaterium obtained from other sources also proved negative, pointing out high specificity of antigenic properties of different phages for B. megaterium.

In our preliminary experiments, the complement fixation reaction did not give unequivocal results in relation to phage specificity.

Experimental results pertaining to the role of temperature on cultivation, nutrient media and test culture on lytic properties of bacteriophages No. 16 and "Kiev" are presented in Table 2.

Determination of lytic properties was carried out by dropping a particular phage lysate on freshly prepared test cultures inoculated on different media. Experimental results were read after 24 hr of incubation at the indicated temperature.

The results presented in Table 2 show that phages No. 16 and "Kiev" differ from each other in several properties. Thus, phage No. 16 did not lyse the sensitive culture FChA on Hottinger's medium at either 26 or 37 C. Phage "Kiev" did not lyse sensitive cultures No. 2 and FChA on Hottinger's medium at 37 C. At 26 C this phage completely lysed culture FChA and culture No. 2 only weakly. On peptone-com agar phage No. 16 caused complete lysis of FChA with formation of secondary growth at 26 C and 37 C, and lysed culture No. 2 at 37 C. In some experiments phage No. 16 did not lyse culture No. 2 at 26 C, and in others caused partial lysis of this culture.

TABLE 2. Influence of Nutrient Media and Temperature of Cultivation on the Lytic Properties of Phages No. 16 and "Kiev"

Test culture	Medium	Phage No. 16		Phage "Kiev"	
		26 C	37 C	26 C	37 C
<i>B. megaterium</i> strain FChA	Hottinger's	No lysis	No lysis	Complete lysis	No lysis
	Peptone-corn	Lysis, many secondary growth colonies	Lysis, many secondary growth colonies	Complete lysis, single secondary growth colonies seldom seen	Complete lysis, single secondary growth colonies seldom seen
	Yeast-casein	Incomplete lysis	Lysis, secondary growth colonies	Complete lysis, no secondary growth colonies	Complete lysis, no secondary growth colonies
<i>B. megaterium</i> strain No. 2	Hottinger's	No lysis	No lysis	Partial lysis, poorly expressed	No lysis
	Peptone-corn	None or partial lysis	Lysis, secondary growth	Complete lysis, occasional single secondary growth colonies	Complete lysis, occasional single secondary growth colonies
	Yeast-casein	No lysis	Partial lysis, expressed by individual plaques	Complete lysis, no secondary growth colonies	Complete lysis, no secondary growth colonies

On yeast-casein medium phage No. 16 was weakly lytic for culture FChA at 26°C and produced complete lysis of this culture with subsequent secondary growth at 37°C. Phage No. 16 did not lyse culture No. 2 on yeast-casein medium at 26°C and produced single plaques at 37°C.

Phage "Kiev" completely lysed cultures No. 2 and FChA on yeast-casein medium at 26°C and 37°C.

Consequently, phages "Kiev" and No. 16 differ from each other in regard to media and temperature. It is important to point out that lytic activity of phage No. 16 was more extensive at 37°C than at 26°C, particularly when tested on culture No. 2. As has been shown previously (Rautenshtein et al., 1960), culture No. 2 readily produces variants sensitive to phage No. 16. It is possible that more variants are produced at 37°C than at 26°C.

In connection with the established differences in lytic and serological properties of phages "Kiev" and No. 16 it was interesting to determine the individuality of these phages when titrated by an overlay technique using different lower and upper media and with different test cultures.

Table 3 indicates the effect of medium on titration of phage "Kiev," and Table 4 shows the effect of indicator cultures and the medium on results of titration of phage No. 16. From the data in Table 3 it is seen that composition of medium has a decided effect on phage concentration. Hottinger's medium, not suitable for lysis by

phage of sensitive cultures of *B. megaterium*, markedly lowers this activity even when it forms only the lower layer. Apparently this medium contains substances inhibitory for certain phages of *B. megaterium*.

The data in Tables 3 and 4 show that the most favorable medium for titration of experimental phages is peptone-corn agar, when it is used either as top or bottom layer.

The composition of medium also significantly influences plaque size. The largest plaques of phage "Kiev" are observed on peptone-corn agar and yeast-casein agar (Figs. 1 and 2 and Table 3). Plaque size is drastically decreased with the use of Hottinger's medium as lower layer (Fig. 4). The temperature of incubation also influences markedly plaque size. In growing phage "Kiev" at 37°C the plaque size is significantly less than at 26°C (Fig. 3).

However, data pertaining to the effect of indicator culture on titration results are of particular interest. It was found that in titration of phage No. 16 on *B. megaterium* strain mutilate, the titer was significantly higher (about 10 times) than in titration on cultures of FChA and KM (Table 4).

SUMMARY

1. A comparative study of two phages of *Bacillus megaterium* No. 16 and "Kiev" isolated upon phagolysis of *B. megaterium* strain No. 2 during the preparation of

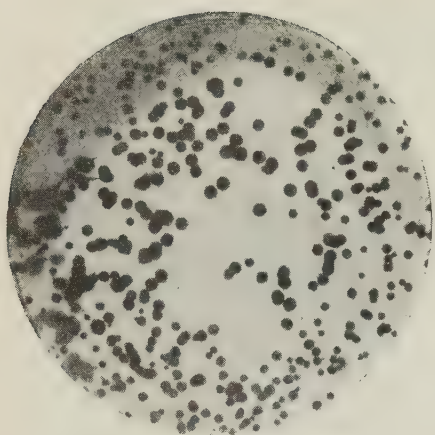


Fig. 1. Plaques of phage "Kiev" on peptone-corn agar at 26°C. Magnification 8:9.

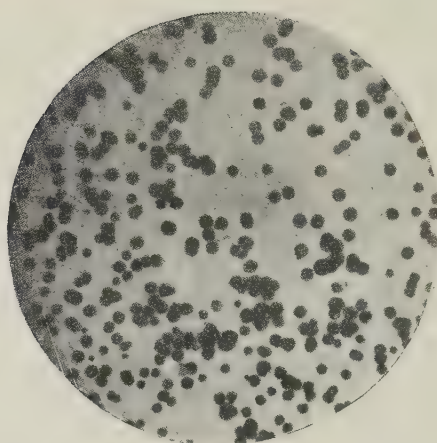


Fig. 2. Plaques of phage "Kiev" on yeast-casein agar at 26 C. Magnification 8 : 9.

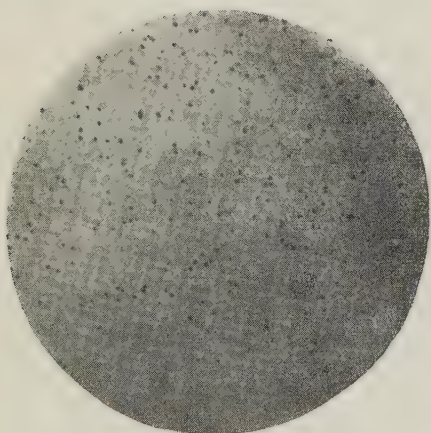


Fig. 3. Plaques of phage "Kiev" on peptone-corn agar at 37°C. Magnification 8:9.

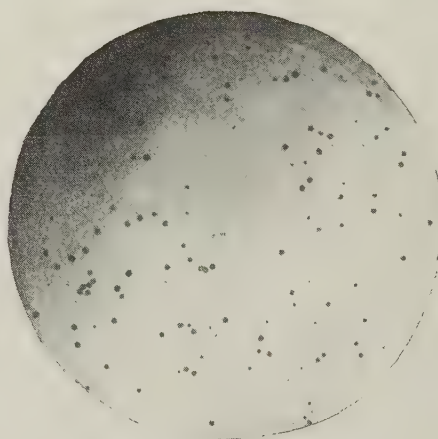


Fig. 4. Plaques of phage "Kiev" at 26°C: lower layer - Hottinger's medium, upper layer - peptone-corn agar. Magnification 8 : 9.

TABLE 3. Effect of Composition of Nutrient Medium on Titration of Phage "Kiev" Using Overlay Technique on B. megaterium strain FChA

Medium		Expt. No.	Number of plaques after using 0.5 ml of phage diluted to		Plaque diameter, in mm
lower layer	upper layer		10^{-9} *	10^{-9} *	
Hottinger's	Peptone-corn agar	1	174	20	1-1.3
		2	193	26	1-1.3
Peptone-corn agar	Peptone-corn agar	1	500	57	1-3
		2	473	47	1-3
Yeast-corn agar	Yeast-corn agar	1	442	46	1-3
		2	481	56	1-3

* Identical dilutions indicated in Russian original—Publisher.

TABLE 4. Effect of Nutrient Medium and Test Culture on Titration of Phage No. 16 of B. megaterium

Test culture	Medium		Average number of plaques after growth on 0.5 ml of phage diluted to			
	upper layer	lower layer				
			10^{-5}	10^{-6}	10^{-7}	10^{-8}
<u>B. megaterium</u> strain FCh A	MPA	PCA	23	1	0	0
	PCA	PCA	74	11	0	0
<u>B. megaterium</u> strain KM	MPA	PCA	23	2	0	0
	PCA	PCA	84	3	1	0
<u>B. megaterium</u> strain mutilate	MPA	PCA	876	87	8	0
	PCA	PCA	870	81	6	1

phospho-bacterin under industrial conditions showed that these phages differ serologically (in the neutralization reaction), in their lytic capacity in defined media, as well as in their temperature requirements.

2. These data as well as those of preceding studies showing morphological differences between the particles make it quite certain that phages No. 16 and "Kiev" are not genetically related to one another.

3. The most favorable medium for the manifestation of the lytic properties of the above phages as well as for titration of them by the overlay technique is peptone-corn agar.

In Hottinger's medium phage No. 16 did not lyse susceptible bacteria either at 26 C or at 37 C; the "Kiev" phage lysed only slightly at 26 C and not at all at 37 C.

Even the use of Hottinger's medium for titration by the overlay technique as the lower layer medium caused a drastic decrease of the titers as well as of the size of plaques. There are apparently some substances in Hottinger's medium which inhibit the phages of B. megaterium.

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BIOLOGICAL CHARACTERISTICS OF STREPTOCOCCAL PHAGES

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Only limited information is available regarding streptococcal phages active on group A hemolytic streptococci. At present, the published data do not allow differentiation of individual strains of streptococcal phages on the basis of variation in their biological properties. However, it is clear that solution of the question pertaining to rational classification of phages, as seen from definitive studies of the T system of phages (Adams, 1959), as well as of Newcastle disease phages (Ershov, 1959), will certainly be helpful in determination of many theoretical and practical questions connected with the phenomenon of bacteriophagy.

The present study is devoted to investigation of biological properties of streptococcal bacteriophages which may possibly form the basis for their classification. For this purpose the following were established: plaque morphology, serological properties of phages, their stability to inactivating action of temperature, urea, sodium citrate and trypsin, ability to adsorb on sensitive bacterial culture, the range of lytic activity of phages, and properties of secondary cultures.

Five streptococcal phages active on group A hemolytic streptococci were used in this study. Of these, phage SA1 was received from the collection of the Department of Microbiology of Leningrad Sanitary-Hygienic Medical Institute; phages SA2, SA3, and SA4 were

isolated by us from bacterial cultures; phage SA5 was isolated from a mouse infected with a streptococcus.*

Four indicator strains were used in this study. Of these, 2 strains, 4S and 4A, belonged to Griffith's serotype 4, and two others, 1SB and 1R, to serotype 1. Strain 4S was utilized for study of phage SA1, strain 4A was used for identification of phages SA2 and SA3, and strains 1SB and 1R were used in the same connection with phages SA4 and SA5.

The above-enumerated phages may be divided into 4 groups according to morphology of their plaques. The plaque size varied from 0.25 to 0.55 mm, which exemplifies their small size in comparison with coliphages. All phages, with the exception of SA1, were characterized by plaques with secondary growth, differing in nature with different phages (Fig. 1).

Serological characteristic, indicated by cross-neutralization reactions, is one of the more important criteria for classification of phages.

Neutralization reaction was carried out according to a well established method (Adams, 1950) and the ob-

* Phages SA1, SA2, SA3, SA4, and SA5 were described in previous publications as 4T, 1399 (4A, 1657), (4A, 1383), (1SB and 1R) and 1R.

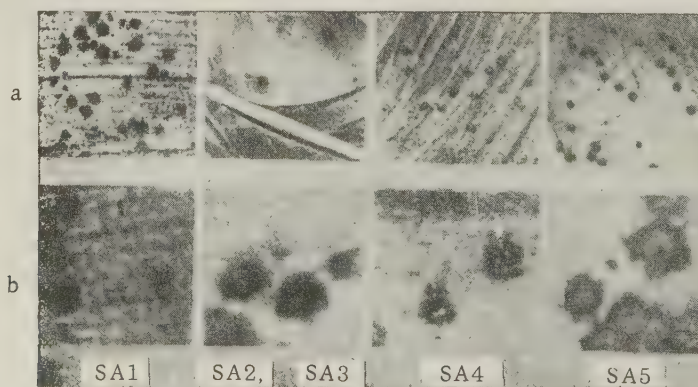


Fig. 1. Morphology of plaques of streptococcal phages. a) Plaques of each phage on dishes; b) the same enlarged 22 x.

TABLE 1. Serological Characteristics of Streptococcal Bacteriophages

Phage	Serological group	Numerical value of neutralization constant of phages by antiphage sera (in min ⁻¹)			
		SA1	SA2	SA3	SA4
SA1	I	28.7	0	0	0
SA2	II	0	43.1	38.5	0
SA3	II	0	42.6	38.0	0
SA4	III	0	2.0	1.5	19.0
SA5	III	0	0	0	7.8

tained results were utilized for determination of constant of speed of phage neutralization according to the following formula:

$$K = \frac{2.3 \times D}{t} \times \lg \frac{P_0}{P},$$

where K = neutralization constant (in min⁻¹); P₀ = concentration of phage at beginning of experiment; P = concentration of phage after t minutes; D = dilution of serum.

Data presented in Table 1 point out the difference in antigenic structure of phages, according to which they may be divided into 3 groups. Group I consists of phage SA1, group II includes phages SA2 and SA3, and group III consists of phages SA4 and SA5. Low titer of the latter phage did not allow production of specific immune serum; consequently, there are no data covering this point in the table.

The results of neutralization tests agree with experimental results of agglutination of bacteria with adsorbed phage in antiphage serum, carried out according to the method proposed by Burnet (Burnet, 1933, a, b; Burnet et al, 1937).

Thermostability of phages was determined according to Friedman and Cowels' method (Friedman and Cowels, 1953). It became apparent that all phages, except for phage SA5, were inactivated to a considerable degree (89-99%) within 20 min at 65 C. In these cases there existed considerable correlation between the morphology of plaques and thermostability of phages on the one hand, and their serologic characteristics on the other hand. The data in Fig. 2 indicate the differential sensitivity of phages of Group III to a temperature of 65 C. Thus, phage SA5 was the least stable, in contradistinction to phage SA4, which was inactivated only at 75 C. Of the remaining phages, phage SA1 was the most stable, and phages of serogroup II were intermediary in nature. It was observed that the differences in thermoinactivation of phages are more pronounced, the higher the heating temperature (Table 2).

Resistance of streptococcal phages to action of concentrated solutions of urea (8-8.6M) was determined

using Burnet's method (Burnet, 1933, a, b). Data presented in Fig. 3 point out strong stability of phages of serogroup II to this agent and strong sensitivity of phage SA1 after 60 min contact with urea, while phages of serogroup III were divided into 2 subgroups: phage SA5 was partially inactivated by urea, and phage SA4 was stable toward its activity.

Action of sodium citrate on phages was studied according to Burnet's technique (Burnet, 1933, a, b). It has been shown that all streptococcal phages are stable to action of this substance in its concentration in agar from 0.125 to 1.25% (Table 3).

Taking into consideration the differences of reported data pertaining to action of proteolytic enzymes on phages and, in particular, that of trypsin (Arnold and Weiss, 1925; Northrop, 1938; Kalmanson and Bronfenbrenner, 1943), we attempted to observe the influence of the latter on streptococcal phages. Experiments were carried out according to procedures suggested by Adams for neutralization reaction of antiphage serum. We used solutions of trypsin (Difco) in broth in concentrations from 0.01 to 2.5 mg/ml. Only phage SA1 proved sensitive to trypsin; 40-75% was inactivated in 30 min at 37C at an enzyme concentration of 0.83 to 2.5 mg/ml. Trypsin concentration of 0.1 mg/ml and less had no significant effect on phage SA1. Solutions of trypsin heated at 72C for 60 min lost activity in relation to this bacteriophage.

Adsorption activity of phages is characterized by constant K of speed of adsorption on sensitive bacterial cells. Other conditions being equal, this measure serves as a convenient criterion for evaluation of their activity. Bacteriophages active in relation to any given microbial species may be differentiated according to speed of adsorption, which is considered as dependent on their lytic activity (Li Khuan-lo, 1958). Experiments were carried out according to generally accepted methods (Adams,

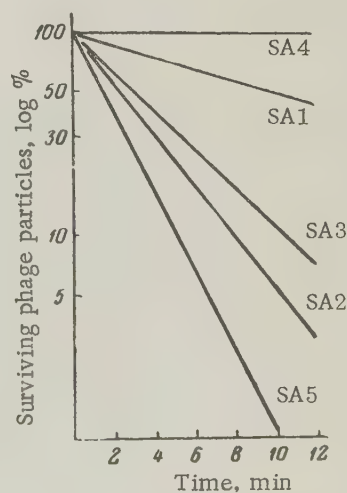


Fig. 2. Inactivation of streptococcal phages at 65C.

TABLE 2. Properties of Streptococcal Bacteriophages

Phage	Sero-logical group	Plaque morphology	Inactivation constants (in min ⁻¹) at temperature			Sensitivity to action of sodium			Constant of rapidity of adsorption in ml/min	Results of cross resistance of cultures	Virulence
			60°	65°	75°	urea	citrate	trypsin			
SA1	I	With sharply inscribed borders, without secondary growth	0.024	0.073		0.038	—	++	3.2×10^{-10}	Absence of cross lysis	Virulent
SA2	II	Cloudy with solid center of secondary growth	0.078	0.27		0.001	—	—	1.1×10^{-10}	Same	Moderate
SA3	II	Same	0.078	0.29		0	—	—	1.3×10^{-10}	"	Same
SA4	III	With inscribed borders and several centers of secondary growth	0	0.007	0.43	0	—	—	2.2×10^{-10}	"	"
SA5	III	With diffuse secondary growth, surrounded by clear zone and in a series of cases with central clearing	0.090	0.12		0.006	—	—	1.0×10^{-10}	"	"

1950) utilizing 18-19 hr broth cultures. It was shown that the greater number of phage particles was adsorbed on sensitive cells during the initial 10 min; subsequently, the amount of adsorbed phage continued to increase less rapidly. The pertinent data are presented in Fig. 4.

As can be seen from the indicated data, phage of serogroup I was adsorbed on cells more actively than phages of serogroup II. As for phages of serogroup III, phage SA4 is closely related to phage SA1, and phage SA5 to phages of serogroup II. The differences appear clearer in comparing the average adsorption constants (Table 2).

In studying the adsorption activity of phages, we considered that of all phases of interrelation of phage with bacterial cell, this phase holds the greatest significance for their characterization. Investigation of other phases and specifically of the latent period of phage development is a problem for future study.

The range of phage activity was studied on 165 strains of group A hemolytic streptococci by dropping phages on solid nutrient media (Table 3). It was possible to group streptococcal cultures according to their sensitivity to phages of different serological groups. Thus, 95 strains were sensitive to phage SA1, and 13 to phages of serogroup II, 21 to phages of serogroup III, with complete absence of cross lysis of cultures. The remaining 36 strains were resistant to phage. These data support serological differentiation of phages. On the other hand,

they do not allow establishing dependence between serotypes of streptococcal cultures and their sensitivity to phages.

Thus, for example, phage SA1, multiplying on a strain of serotype IV, showed greatest activity in relation to cultures of the same type and weakly lysed strains of heterologous types. Only undiluted phages SA2 and SA3 lysed most cultures of serotype I, while strains of serotypes 4, 24, 26, 29, of similar antigenic composition (Ioffe, 1948), are lysed by phages diluted to 10^{-4} and 10^{-6} . On the other hand, phages SA4 and SA5, grown on strains of serotype I, lysed equally well all sensitive cultures regardless of their type.

TABLE 3. Grouping of Strains of Group A Hemolytic Streptococci According to their Sensitivity to Different Phages

Group	No. of strains	Bacteriophages				
		SA1	SA2	SA3	SA4	SA5
I	95	+	—	—	—	—
II	13	—	+	+	—	—
III	21	—	—	—	+	+
IV	36	—	—	—	—	—

Note: + sensitive; — not sensitive

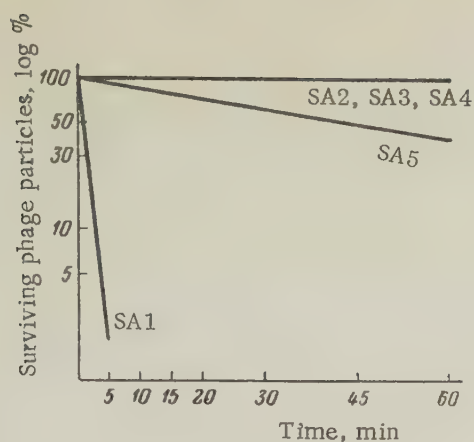


Fig. 3. Action of urea on streptococcal phages.

In investigating cross-sensitivity of secondary cultures to phages, it was established that they are stable to action of all phages. It is natural that these data may not serve as criteria for differentiation of phages. Luria and Hershey (Ershov, 1959) also point out the significant relation of this property for classification of bacteria viruses.

It has been possible to show that resistance of secondary cultures, developed in the course of action of phages of serogroups II and III, to homologous phages is explained by lysogenic state of these cultures. Exposure of these secondary cultures to ultraviolet light from bactericidal lamp BUV-30 for 30-120 seconds resulted, in all cases, in production of corresponding phage. This allows relegation of above agents to the category of temperate phages (Table 2).

In this manner, we determined a series of biological characteristics of streptococcal bacteriophages not only by studying the properties of extracellular phage (serological properties, resistance to inactivating agents), but also its interaction with the microbial cell (plaque morphology, adsorption of phage to cell, range of action, cross-stability of secondary cultures). Therefore, it appears to us that, without attempting exhaustive biological characterization of streptococcal phages, the obtained data may be considered as a basis for their classification. In this connection, one must consider that for inclusion of any two phages in one and the same group it is sufficient to have similarity of two characteristics, of which one must be leading in nature (Adams, 1959). Therefore, basing classification of streptococcal phages on their serological properties one can subdivide the investigated phages into 3 groups, in which case serogroup I is most clearly differentiated. Of considerable interest is serogroup III, marked by great diversity of properties of the component phages; however, similarity of serological

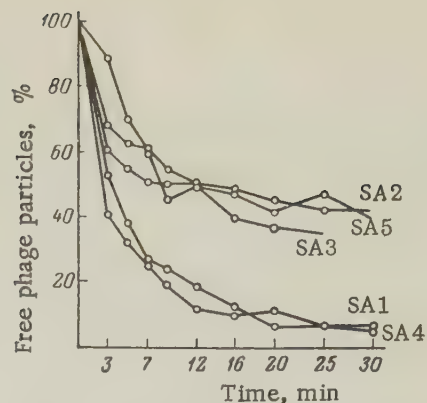


Fig. 4. Adsorption of streptococcal phages on bacterial cultures of sensitive cultures.

properties and the range of action of phages SA4 and SA5 allowed their inclusion into the same group (Table 2).

SUMMARY

1. The classification of streptococcal phages, like that of other bacterial viruses, can only be based on a detailed study of their biological properties.
2. It was possible to divide 5 streptococcal phages into 3 groups differing from each other in complexes of biological properties.
3. These phages can be considered as standards for comparison of freshly isolated phages active on group A hemolytic streptococci.

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EFFECT OF WATER VAPOR ON *Actinomyces violaceus* AERIAL MYCELIUM CELL WALL

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The use of top light and polarized top light in studying the aerial mycelium structure of actinomycetes (Krasil'nikov and Kalakutskii, 1959) has led us to presume that the mycelial wall is heterogenous (Kalakutskii and Sokolov, 1961). Here we report the effect of water vapor on parts of the aerial mycelium surface of *Actinomyces violaceus* strain 829. These surface parts possess the property of yielding a colored reflection in top light, they are optically anisotropic in reflected polarized light (crossed polaroids), and they stain with Sudan B.

METHODS

A. violaceus strain 829 was grown for four days at 27°C on cellophane sheets overlying potato agar. Then the cellophane with the actinomycete culture was removed and kept for one day at room temperature in a desiccator over a saturated NH_4Cl solution (the relative humidity of the air over this solution was 79%). After this we prepared impression smears on grease-free slides.

The examination and photography of the smears were conducted with the MBI-6 microscope. For top light examinations we used the 40X epi-objective; for transmitted light we used the apochromatic 90X objective.

For staining with Sudan B we used a saturated alcohol solution of the dye, diluted two-fold with *n*-butanol. The slides were stained for 5 hr at room temperature. The stained preparations were mounted in water. The experiments showed that in evaluating the effects of various agents on cell wall structure, it is essential, during the various processings of the slide, to observe always the same cell or group of cells, fixing the position with a micrometer scale table. The aerial mycelial actinomycete cells are hydrophobic; when a slide is flooded with water a large number of the cells, often in the form of aggregates, is washed off the glass and floats to the surface. If the slide is then taken out of the water, a part of these cells may again adhere to the glass, creating the false impression that water has no significant effect on these cells. This, as we shall see, is far from being so.

RESULTS

Our observations showed that keeping unfixed or heat-fixed impression smears for 24 hr in an atmosphere

of 2% osmic acid vapors and 40% formaldehyde removes the "florescent" effect of aerial mycelium cells in top light and destroys the optical anisotropy of the cell surface in polarized top light. Slides placed in a desiccator over distilled water served as the control in these experiments. As it turned out, placing the slides in water vapor at room temperature for a period of a day likewise completely removed the "florescence" and optical anisotropy. Thus, this shows that the action of the osmic and formaldehyde solution vapors was unspecific in this case.

To confirm that the changes were actually caused by the effect of water vapor, we ran a series of experiments, placing the slides (for one day at room temperature) in desiccators over saturated solutions of compounds possessing constant relative humidities as follows: $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$ —98%; K_2HPO_4 —92%; NH_4Cl —79.5%; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ —35%.*

We found that keeping the slides over solutions of $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$ and K_2HPO_4 had the same result as keeping them over distilled water. Solutions of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and NH_4Cl had no effect, or an insignificant one. Thus, one might assume that a relative atmospheric humidity of over 80% represents that "threshold" beyond which, during the given reaction time, water vapors act on the actinomycete aerial mycelium cell wall surface to remove its "florescent" effect in top light and its optical anisotropy effect in polarized top light (crossed polaroids).

When we increased the reaction time of the slides over saturated solutions of the above compounds to 2-3 days, the amounts of water vapor present over the NH_4Cl and $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ solutions became sufficient to cause similar effects in a number of cases. No effect was observed, however, when the slides were placed for long periods (up to 5 months) over desiccating substances such as anhydrous CaCl_2 and P_2O_5 . For this reason, it is practically feasible to store the slides over these substances.

*Data presented from The Chemist's Reference [in Russian] (Goskhimizdat, 1961) Vol. 1, p. 48.

When the impression smears are kept over distilled water for 3-8 days, a dissolution of the mycelial cells usually takes place. In unfixed slides this process is not-ably more rapid than in heat-fixed ones. It is interesting to note that formed spores are not subject to such a dissolution.

In the next series of experiments we studied the time in which the water vapor in the distilled water at- mosphere exerts its action on the cell wall surface at room temperature. We established that a 5 min con- tact significantly reduced, while a 10 min contact al- most completely eliminated, the "florescence" effect, the optical anisotropy, and also the cell wall's staining properties for Sudan B.

Figure 1 shows a group of A. violaceus cells on an impression smear, heat-fixed. We see a localized "flo- rescence" of cells (the colored subject was photographed on black and white film). Figure 2 shows the same cells in polarized top light. We noted an exact correspondence of cell wall structures in these two methods of observation. The slide with these cells was then placed over distilled water in a desiccator for 3 min. Figure 3 shows the same cells after their sojourn in a damp atmosphere, as photo- graphed in top light (colored film); Figure 4 shows them in a polarized top light. As is seen, a part of the cell walls had lost their ability to give the "florescent" effect and optical anisotropy after 3 min over distilled water. It is interesting to note that those parts of the cell walls which had lost the above properties had concomitantly lost their stainability with Sudan B, as is seen in Fig. 5, which shows the same cells as Figs. 3 and 4, stained with Sudan (photographed in transmitted light).

The water vapor action on the above-mentioned properties is irreversible. Subsequently placing the slides over calcium chloride or warming them did not restore to the cell wall structures their lost properties.

When slides are prepared in similar manner, we find an analogous reaction to water vapor in the cell wall surface structures of aerial mycelia of Actinomyces coelicolor strain 206, Actinomyces albidoflavus, Actin- omyses sp. 1276 (related to Actinomyces lavendulae).

The time required for the effect to manifest itself, however, may vary somewhat.

Submerging the slides in water for 5-10 seconds has the same effect on cell wall structure as contact with the water vapor.

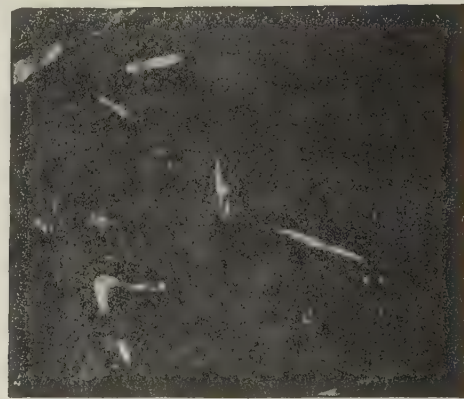
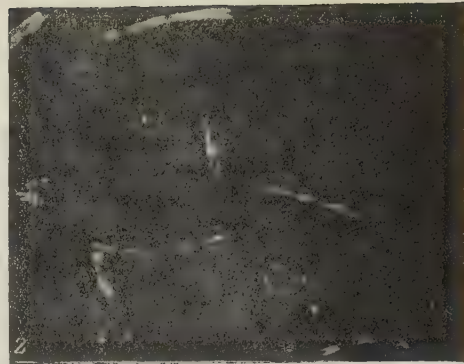
It would be interesting to find out what causes this high reactivity of aerial mycelium cell wall structures to water vapor. The answer to this question is not clear. One might only presume that the water molecules bind up with the molecules of a substance or substances loca- lized in the cell wall of the actinomycete aerial myce- lium, and responsible for the cell wall's specific beha- vior in top light. There occur, with this, both a struc- tural change (disappearance of optical anisotropy) and a blocking of those receptors which, possibly, are re-

sponsible for binding the dye molecule (Sudan B) out of its solution.

Our procedure for processing materials for the above- described experiments (keeping cellophane sheets with the developed actinomycete colonies over a saturated NH_4Cl solution for 24 hr) was chosen empirically. As we discovered in further experiments, this procedure in itself sensitizes the cells, raising their susceptibility to water vapor. In a series of experiments, Actinomyces violaceus was grown on cellophane sheets overlying pota- to agar. After 4 days' incubation at 28C, we proceeded as follows: in series I we left the cellophane with the actinomycetes for an additional day on the agar; in se- ries II we removed the cellophane with the culture and incubated it over distilled water for 24 hr; in series III the cellophane was removed and incubated for 24 hr over a saturated NH_4Cl solution; in series IV the removed cellophane was incubated for 24 hr over anhydrous P_2O_5 . The incubation temperature was 28C in all cases. In all four cases we prepared impression slides of the col- onies and heat fixed them. These slides were then sub- jected to water vapor action at room temperature for varying periods of time. It was definitely established that cells of series III and IV were much more "sensitive" to water vapor; a 10-15 min contact with distilled water vapor changed the behavior toward top light of a vast majority of cells. The same reaction time proved much less effective for cells of series I and II. Sensitivity of cells in series I, II, III and IV to water in the liquid phase (submergence in bidistillate for varying periods) showed no appreciable differences. From these results we might presume that the sensitivity of cell wall struc- tures to water vapor action is connected with the physio- logical state of the culture, and is heightened upon in- cubation of the cultures in conditions of lowered humidi- ty. We should remember that a coloration of the actino- mycete aerial mycelium hyphae with various interferen- tial colors could be observed directly in the agar colonies in top light. The development, in this case, had oc- curred in a relative atmospheric humidity approaching 100%. It is interesting, likewise, to note that in the impression slides the cells located singly were more "sensitive" to water vapor and liquid water action than were the cells in clusters.

When the impression slides are fixed over a burner, vapors of water condensation may settle on the slide. The subsequent evaporation of this moisture may change the properties of the cells. Therefore, it is better to pre- pare the impression smears on warm slides. If the ac- tinomycete slides are prepared from agar or cellophane which has not been incubated in a reduced humidity atmosphere, one should likewise avoid all contact of water with the slide.

In conclusion, I thank Professor N. A. Krasil'nikov for his supervision of the above research, and also A. A. Sokolov for his assistance in the work.



Figs. 1 to 5. Aerial mycelium cells of 5-day-old A. violaceus culture. Impression smear. Heat fixation. Explanations in the text.

SUMMARY

A short exposure of unfixed or heat-fixed impression slides of actinomycete aerial mycelium cells to water vapor causes the cell wall structures to lose their interference coloration effect in top light, their ability to show optical anisotropy in polarized top light, and their stainability with Sudan B. A preliminary incubation of

the growing culture in a lowered humidity atmosphere increases the cell wall's sensitivity to water vapor.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

CHEMICAL COMPOSITION AND AGGREGATING CAPACITY OF THE MICROBIAL MASS

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Polyuronides are quite widespread in nature. They enter into the composition of plants and organic substances of soil.

The polyuronides play a definite role in the formation of soil structure (Fiedler, Bergmann, 1954, 1955; Martin, 1946; Qastel, 1952).

The high soil content of polyuronides and their possible part in the improvement of soil structure instigated a search for the synthesis and accumulation of this group of compounds.

Rudakov (1949 a, b) and Rudakov and Birkel' (1949) consider that an active compost is formed from pectinous substances of plants and from uronic acids of microbial origin.

Root pectins are split down to uronic acids even during the plant's life, owing to the action of protopectinase bacteria. The accumulating uronic acids, in combination with microbial proteins, form the urono-proteid complex, which possesses the qualities of an active compost. Thus Rudakov explains the well known fact of the formation of firm soil structure during the growth periods of perennial grasses, while the decomposition processes of dead root masses are not yet very active.

According to Novogrudskii (1959), Rudakov's hypothesis has great significance for understanding the structural formation mechanism of soil.

In this connection, an investigation of the role of polyuronides in the structural formation of soil assumes a theoretical and practical interest.

The aim of the present work was to determine the dependence of the aggregating capacity of the microbial mass on its uronic acid content.

METHOD

From the rhizospheric soil of esparsette in its second year of life, we isolated pure cultures of the microorganisms Bacillus polymyxa, Azotobacter chroococcum, Aspergillus niger, and also a Bacillus subtilis-Bacillus mesentericus group (we did not separate the cultures of this group.)

For accumulating a biomass, A. chroococcum was grown on Ashby's agar medium at 26-27°C for 48 hr; the cultures of the subtilis-mesentericus group and the B. polymyxa were grown in peanut-peptone sugar broth at 24-25°C for 66 hr; the A. niger - in fluid medium of the following composition: $C_6H_{12}O_{11}$ -5%; NH_4NO_3 -0.3%; KH_2PO_4 -0.1%; $MgSO_4$ -0.1%; $FeSO_4$ -2 drops of a 1% solution - this at 30-31°C for 66 hr.

The grown bacterial biomass was separated from the medium and analyzed. The A. niger films were thoroughly washed with distilled water. Total nitrogen and protein nitrogen contents were determined by the micro-Kjeldal method (Belozerskii and Proskuryakov, 1951). Nonprotein nitrogen was calculated by the difference between the total and protein nitrogen. Protein was calculated by multiplying the protein-nitrogen figure by the coefficient 6.25; total carbon content was estimated by Turin's method (1954); the amount of uronic acids - by Shumka and Balabukh's modification of the Tollens - Lefevre method.

The analyses were made in triplicate. Results were calculated as percentages of absolute dry microbe mass substance (Tables 1 and 2).

From the data presented we see that the chemical compositions of the various microbe masses varied.

TABLE 1. Nitrogen Content as Percentage of Absolute Dry Microbial Mass Substance

Microorganism	Total nitrogen	Nonprotein nitrogen	Protein nitrogen	Protein	Protein nitrogen, as % of total nitrogen
<u>B. polymyxa</u>	9.13	5.77	3.36	21.00	36.80
<u>A. chroococcum</u>	5.52	2.21	3.31	20.69	66.76
<u>A. niger</u>	7.00	3.76	3.24	20.25	46.28
<u>Subtilis-mesentericus</u> group	11.14	3.88	7.26	45.37	64.47

TABLE 2. Carbon and Uronic Acid Contents in % Absolute Dry Microbial Mass Substance

Microorganism	Total carbon	Amount of uronic acids	Uronic acid carbon	Uronic acid carbon as % of total C	Correlation between uronic acids and protein
<i>B. polymyxa</i>	39.39	15.60	5.79	14.72	0.74
<i>A. chroococcum</i>	41.71	13.99	5.22	12.53	0.67
<i>A. niger</i>	48.00	11.45	4.25	9.09	0.56
<i>Subtilis-mesentericus</i> group	40.86	9.55	3.54	8.66	0.21

TABLE 3. Yield of Water-Stable Aggregates (in percentages)

Microorganism	1-0.5 mm fraction	Fraction over 1.0 mm	Total sum of water-stable aggregates > 0.5 mm
<i>B. polymyxa</i>	1.0	68.6	69.6
<i>A. chroococcum</i>	3.2	46.5	49.7
<i>A. niger</i>	2.1	38.1	40.1
<i>Subtilis-mesentericus</i> group	0.4	4.4	4.8

The culture of the *subtilis-mesentericus* group was the highest in total and protein nitrogen content.

The *A. chroococcum*, however, had the highest protein nitrogen:total nitrogen ratio.

By carbon content the tested cultures assumed the following order: *A. niger*, *Azotobacter*, cultures of the *subtilis-mesentericus* group, *B. polymyxa*.

B. polymyxa had the highest uronic acid content (15.60%); the *subtilis-mesentericus* group had the lowest (9.55%).

In their uronic acid carbon ratio to the total carbon content, the cultures fell into the same order as for uronic acid content.

We then tested the aggregating capacity of the microbe mass of these cultures.

As substrate we used a mixture of clay and sand, which was baked in a furnace, sifted through a sieve with a hole diameter of 0.5 mm. This mixture was devoid of structure, organic matter and microflora.

The microbial mass was destroyed by thorough grinding with sand, and was then made into aqueous suspensions.

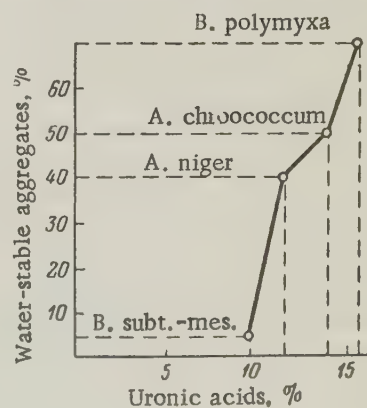
The mixture was moistened in the proportion of 3 g of bacterial suspension to 15 g substrate. Aggregation was produced by thorough mixing. The vessels with aggregates were kept at room temperature for 48 hr. The water-stability of the formed structure was determined by Tulin's method, performed in triplicate. The wet mixture was spread on sieves with hole diameters of 0.5 mm and 1 mm. Using water for the mixture in the same

procedure, the yield of water-stable aggregates was zero (Table 3).

The data of Table 3 show that the highest aggregating effect was given by the microbe mass of *B. polymyxa*, whereas the culture of the *subtilis-mesentericus* group gave the lowest.

Comparing data of the chemical and mechanical analyses reveals a connection between the quantitative uronic acid content of the microbe mass and its aggregating capacity. This relationship is demonstrated graphically (see figure).

It is essential to note the unproportionality between the water-stable aggregate yield and the quantity of uron-



Dependence of water-stable aggregate yield on uronic acid content.

ic acids. Apparently, the complicated process of soil structure formation cannot be accounted for by the action of any one group of chemical compounds.

Since Rudakov's hypothesis considers the uronoproteid complex to be an active compost, we calculated the correlation between uronic acids and protein. The lowest correlation proved to be in the subtilis-mesentericus group culture; in B. polymyxa the correlation approached unity.

Thus, of the four cultures analyzed, the smallest quantity of uronic acids was found in cultures of the B. subtilis-B. mesentericus group (9.5%). These cultures yielded the lowest aggregating effect (4.8%). The highest amount of uronic acids was found in B. polymyxa (15.6%), which also yielded the highest aggregating effect (69.6%).

In conclusion, we thank O. A. Semikhatova for her great assistance in conducting the microbiological analyses.

SUMMARY

1. The cultures studied, viz., Bacillus polymyxa, Azotobacter chroococcum, Aspergillus niger, and the group of Bacillus subtilis-Bacillus mesentericus, differ from one another in their content of some forms of nitrogen, carbon and uronic acids.

2. The aggregating capacity of the above cultures is connected with the uronic acid content.

3. The greatest aggregating capacity is exhibited by the microbe mass of B. polymyxa, and the least by that of the subtilis-mesentericus group.

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EFFECT OF SOME ANTIBIOTICS ON THE PHYSIOLOGICAL ACTIVITY OF *Azotobacter chroococcum*

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While studying the interrelationships between azotobacter and soil actinomycetes, the necessity arose to determine what effect certain antibiotics produced by actinomycetes have on the activity of the enzymes of respiration and nitrogen fixation.

Of this group of substances that have a great effect on a number of bacteria, the best known are streptomycin, oxytetracycline, chlorotetracycline, and biomycin. However, the nature of their effect on the individual enzyme systems of microorganisms has been studied very slightly, and their effect on the nitrogen-fixing activity of azotobacter has not been studied at all. From the data available in the literature, it can only be concluded that these antibiotics block certain enzyme systems of microbial cells. Thus, for example, according to the data of Umbreit (1949), Smith (1949), and others, streptomycin inactivates the enzymes that participate in the oxidation of pyruvic acid, but according to the data of Roote and Polgase (1955), dihydrostreptomycin inactivates the enzymes which participate in the oxidation of arabinose, lactose, and gluconic acid in *Escherichia coli*. On the basis of his experiments, Belyanski (1954) concluded that the inhibitory effect of streptomycin on the oxidation of a number of organic compounds is connected with the disruption of the synthesis of oxidases and dehydrogenases which contain atoms of metals.

In the presence of this antibiotic, their utilization for enzyme synthesis is disrupted.

Aside from the inactivation of particular dehydrogenases and oxidases, streptomycin can also cause other changes in the biochemical activity of bacterial cells; in particular, it can disturb the synthesis of protein and nucleic acids (Rosenblum and Bryson, 1953; Umbreit, 1953). The nature of this effect of streptomycin is associated with the fact that it produces changes in the structure of the lipid-protein complex of the cell protoplasm and releases nucleic acid (Bukrinskaya and Debova, 1955; Pevzner, 1956; Belozerskii et al., 1956). Therefore, in the protoplasm of bacteria resistant to streptomycin, a decrease in the protoplasmic ribonucleic acid and an increase in deoxyribonucleic acid are observed.

Such are the general data on the effect of streptomycin in bacterial cells. There are only occasional data pertaining to azotobacter (Bershova, 1950; Khirai, 1955), however, to the effect that streptomycin reduces the endogenous respiration of azotobacter if the medium contains nitrates or glutamic acid. Since the addition of peptone reduces this effect, it is concluded that it contains substances which counteract the inhibitory effect of streptomycin on respiration.

The mechanism of action of chlorotetracycline, oxytetracycline, and biomycin has been studied even less. The only data encountered in the literature are those (Jacobson et al., 1954; Johnson and Colmer, 1957; etc.) showing that these antibiotics also inactivate certain dehydrogenases and enzymes that participate in the transfer of the energy of high-energy phosphate bonds.

The effect of chlorotetracycline on these enzymes can be judged on the basis of the following data: at a concentration of $2 \cdot 10^{-4}$ M, it completely inhibits the respiration of *Azotobacter vinelandii*. The mechanism of this inactivation is apparently based on the binding of magnesium atoms which are included in the composition of some respiratory enzymes, because the addition of magnesium salts to the medium sharply reduces the effect produced by this antibiotic.

From the above-stated, it can be seen that the nature of the action of the enumerated antibiotics on bacterial cells has so far been poorly studied. It is this situation that prompted us to undertake the experiments presented, in which the effects of a number of antibiotics on the growth, nitrogen-fixing activity, and respiratory enzymes of azotobacter were studied. In these experiments, along with antibiotics of actinomycete origin, we also used penicillin, the nature of action of which on bacterial cells has been better studied and is associated with the disruption of a number of synthetic processes (nucleic acid metabolism, the synthesis of amino acids and protein, etc.).

Our investigations were carried out with *Azotobacter chroococcum* (strain 53). At first, we studied the effect

* Deceased.

of streptomycin, chlorotetracycline, biomycin, oxytetracycline, and penicillin on the enzyme systems which participate in oxidative processes during respiration. The experiments were set up with thoroughly washed two-day-old azotobacter cells. The cells were grown in Fedorov's modification of nitrogen-free agar medium with glucose; they were washed off with phosphate buffer, and centrifuged. Their washing and subsequent centrifugation was carried out with the same buffer solution.

The appropriate suspension was prepared from cells obtained in this manner. It was aerated for 5 hr so that all of the nutrient reserves in the cells would be oxidized, and was then used in further experiments. The effect of the antibiotics indicated above on the dehydrogenase activity of azotobacter was determined in special Thunberg tubes. The test antibiotics were placed into these tubes in amounts of 10, 100, 1,000, and 10,000 μg per 1 ml. Aside from the antibiotic, 1 ml of methylene blue solution (1 : 10,000), 1 ml of a solution of energy-yielding substance, 3 ml of phosphate buffer (0.1 M), and 2 ml of bacterial suspension were added to the tubes. Air was pumped out of the tubes, and they were placed in an incubator at 32 C. The effect on dehydrogenases of the antibiotics added to the suspension was determined by the rate of decolorization of the methylene blue. As controls, the experiment included tubes with all of the components of the medium, but without a hydrogen donor, and tubes with all of the components of the medium including the hydrogen donor, but with killed azotobacter cells and a ten times more dilute solution of methylene blue. The end of decolorization in the experimental tubes was determined by comparing their color with that of the tube containing diluted methylene blue and killed azotobacter cells. Glucose, mannitol, ethyl alcohol, glycerol, and acetic, butyric, malic, succinic, citric and tartaric acids were used as hydrogen donors. As is well known, all of these compounds are dehydrogenated by means of specific dehydrogenases.

The results of the experiments performed showed that 100 μg of streptomycin depressed the dehydrogenase activity of azotobacter by 30%, 1,000 μg —by 60%, and 10,000 μg —by 70%.

Chlorotetracycline proved to be more active. At 100 μg , it depressed dehydrogenase activity by 70%, and at 1,000 μg —by 100%. Oxytetracycline was less active. At a dose of 100 μg , it had no noticeable effect, but at a dose of 1,000 μg , it depressed dehydrogenase activity by only 10–20%. However, at a concentration of 10,000 μg , it too depressed dehydrogenase activity 100%. Biomycin had an even smaller effect. Even at a dose of 10,000 μg , it only depressed dehydrogenase activity by 20–25%. The same amounts of penicillin had no effect on the dehydrogenation of the hydrogen donors tested. These data show that various antibiotics do not inhibit the dehydrogenase activity of azotobacter to the same extent.

TABLE 1. Effect of Antibiotics on the Consumption of Molecular Oxygen by Washed Azotobacter Cells (in % of control without antibiotic)

Oxidizable substrate	Antibiotic, μg	Streptomycin	Chlorotetracycline	Oxytetracycline	Biomycin	Penicillin
Glucose	0	100	100	100	100	100
	100	35	38	98	72	98
	1000	29	23	91	77	99
	10000	27	19	—	76	97
Mannitol	0	100	100	100	100	100
	100	42	52	79	83	96
	1000	34	—	41	70	85
	10000	26	—	15	66	82
Ethyl alcohol	0	100	100	100	100	100
	100	37	39	75	100	99
	1000	30	—	65	84	86
	10000	30	—	15	82	94
Glycerol	0	100	100	100	100	100
	100	59	44	77	95	96
	1000	48	—	31	78	95
	10000	30	—	18	70	86
Acetic acid	0	100	100	100	100	100
	100	48	51	68	96	97
	1000	40	—	30	79	89
	10000	38	—	15	73	89
Succinic acid	0	100	100	100	100	100
	100	54	70	76	95	92
	1000	34	39	39	83	82
	10000	30	34	23	72	84

Parallel experiments were also set up to study the effect of the enumerated antibiotics on oxidase activity. For this purpose, washed azotobacter cells, together with the hydrogen donor and phosphate buffer, were placed in Warburg vessels and the consumption of molecular oxygen was determined by the manometric method. The results of this experiment are given in Table 1.

The effect of the antibiotics on the consumption of molecular oxygen by washed azotobacter cells proved to be similar to their effect on dehydrogenase activity. They inhibited the consumption of molecular oxygen to approximately the same extent as they did the mobilization of hydrogen. On this basis, it must be concluded that the essence of their inhibitory effect on respiration is primarily connected with their inactivation of the initial stages of oxidation of the substrate, particularly the mobilization and transfer of hydrogen, and not with its binding by molecular oxygen. In our experiments, penicillin had no noticeable effect on the consumption of molecular oxygen by azotobacter either. In the dosages employed, it had no effect on respiratory enzymes.

Because antibiotics of actinomycete origin inhibit the processes of the mobilization of hydrogen, and activated hydrogen is used in azotobacter cells for the

TABLE 2. Effect of Antibiotics on the Reproduction of *Azotobacter* in the Presence of Bound Nitrogen in the Medium (average data of two repetitions; number of cells at start of experiment—5.3 million)

Antibiotic in medium, $\mu\text{g/ml}$	Cells at end of expt., mil.				
	streptomycin	chlorotetracycline	oxytetracycline	biomycin	penicillin
0.0	1006.4	1006.4	1006.4	1006.4	1006.4
0.2	941.0	1120.0	895.0	975.0	984.0
2.0	989.0	878.0	935.0	917.0	904.0
10.0	7.2	7.6	4.8	6.5	888.0
20.0	4.9	5.7	5.0	4.6	7.6
100.0	3.4	3.5	4.5	3.9	4.2
200.0	2.9	3.0	3.1	2.9	2.0

reduction of molecular nitrogen during the course of nitrogen fixation, the natural question arose of precisely what effect the antibiotics under investigation have on the nitrogen-fixing activity of this microorganism under conditions of its usual cultivation.

In order to answer this question, we set up experiments with *A. chroococcum* on sterile nitrogen-free medium (in Fedorov's modification) with glucose, to which sterile solutions of antibiotics in amounts from 2 to 2,000 $\mu\text{g/ml}$ were added. *Azotobacter* was grown in 150 ml Erlenmeyer flasks. Fifty ml of medium containing 1 g of glucose was poured into each flask. After inoculation with *azotobacter*, the flasks were incubated at 30 C for two weeks.

The analyses carried out next showed that small doses of antibiotics had no effect on the efficiency of nitrogen fixation and growth in *azotobacter* (doses of 2 $\mu\text{g/ml}$). In all cases, nitrogen fixation was similar to the control and corresponded to 10-12 mg per 1 g of glucose utilized. Higher doses (100-200 $\mu\text{g/ml}$), however, put a complete stop to the growth of *azotobacter* cells and to the fixation of molecular nitrogen by them. This result can be explained in two ways: either the antibiotics inactivated the enzymes of nitrogen fixation, thus stopping the synthesis of cellular material during the growth of *azotobacter* on medium without bound nitrogen compounds, or they blocked other processes in the synthesis of cellular material and made nitrogen fixation unnecessary. Which of these causes is the more significant can only be decided by means of experiments with a culture of *azotobacter* on nutrient medium containing antibiotics and bound forms of nitrogen.

We set up such experiments on the same nutrient medium, but with the addition of ammonium phosphate calculated to give a carbon to nitrogen ratio of 20 : 1 in the medium. In order to eliminate possible acidification of the medium when using ammonia nitrogen, Sorensen's phosphate buffer was added to it. The experiment on this medium was continued for three weeks and gave the same results as the preceding.

At a concentration of 2 $\mu\text{g/ml}$, streptomycin, chlorotetracycline, oxytetracycline, and biomycin had no noticeable effect on the growth and reproduction of *azotobacter*, but at higher dosages (10 $\mu\text{g/ml}$), they completely stopped its growth in the medium with bound nitrogen as well. On this basis, it should be regarded as established that the nature of the inhibitory effect of these antibiotics is associated, not with the inhibition of the nitrogen-fixing process, but rather with the inhibition of other synthetic processes underlying the de novo formation of the cellular material of the given microorganism. Nitrogen fixation ceases in connection with the absence of cell growth. The data of the experiment in which *azotobacter* was cultured on medium with bound nitrogen is given in Table 2.

In connection with this effect of antibiotics on the growth of *azotobacter*, still another question arose: How do these compounds affect the viability of *azotobacter*?

In order to clarify this question, we made subcultures from the experimental variants in which complete inhibition of growth of the *azotobacter* was observed, and plated them on nitrogen-free agar medium in a Petri dish. The inoculated plates were then kept at 32 C for two days. An examination of these showed rather intensive growth of *azotobacter*. Evidently, the doses of antibiotics that inhibited the growth of this microorganism (20 $\mu\text{g/ml}$ of penicillin and 10 $\mu\text{g/ml}$ of the other antibiotics) were still not bactericidal. The *azotobacter* cells present for several days in the medium with these concentrations of antibiotics remained viable, and grew and multiplied fairly well when transferred to a suitable nutrient medium. This suggests that all of the enzyme systems necessary for the normal functioning of vital processes were completely preserved in them.

If the antibiotic concentrations inhibiting the growth and reproduction of *azotobacter* are compared with the concentrations inhibiting the enzyme systems that participate in respiration, it is easily established that 10-100 times smaller doses are required for the inhibition of growth and reproduction than for the inhibition of respiration. This shows that the mechanism of the inhibitory activity of antibiotics can not be linked with their effect on respiratory enzymes. They primarily inactivate the enzyme systems which participate in the synthesis of cellular material. Therefore, cell growth and reproduction cease at antibiotic dosages which are 10-100 times lower than those required to stop the respiration of resting cells. Only when the doses of these compounds in the medium are higher do they reduce the respiratory activity of resting cells. The nature of this reduction is apparently explained by the fact that the antibiotics react with the proteins that are included in the composition of the corresponding enzymes and alter their state, or else they bind certain groups in their side-chains that are important for the catalytic activity of the enzyme. In either case, the activity of the enzyme has to decrease gradually. When the concentration of the

antibiotic reaches an even higher level, however, such a substantial alteration of the protein portion of the protoplasm evidently begins that it leads to the disruption of the structure of the lipid-protein complex and of its bond with protoplasmic ribonucleic acid. However, these changes are reversible within a certain range, because azotobacter cells which had been kept for several days in an antibiotic solution which completely inhibits their growth and reproduction still remained viable and, when transferred to ordinary nutrient medium, grew normally, carrying out all vital processes including nitrogen fixation.

SUMMARY

1. Streptomycin, chlorotetracycline, oxytetracycline, and biomycin at a concentration of 100 $\mu\text{g/ml}$ inactivate the dehydrogenases of washed azotobacter cells by 50-70 % and prevent the transfer of hydrogen to methylene blue. They also have approximately the same inactivating effect on the utilization of molecular oxygen by washed cells. On this basis, it must be considered that their inhibitory effect on respiration is involved with the initial stages of this process, i.e., with the mobilization and transfer of hydrogen.

2. In growing azotobacter cultures the same antibiotics check growth and reproduction at doses 10-100 times less than those inactivating respiration. Since their effect is quite similar upon azotobacter grown on molecular and fixed nitrogen, it can be inferred that these antibiotics are blocking the enzymes involved in the synthesis of cell substance rather than the enzymes of nitrogen fixation.

3. Exposure of azotobacter cells for several days to a medium containing antibiotics at doses which block

growth and reproduction does not result in death of the bacteria. When subcultured to a normal nutrient medium, they show good growth. Inactivation of some enzymes participating in the synthesis of the cell substance seems to be transient. Upon transfer to a medium containing no antibiotics, their activity is restored so that synthesis of the cell substance does not undergo any appreciable change.

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MICROBIOLOGICAL STUDIES OF CARPATHIAN SULFUR DEPOSITS

IV. Study of Conditions for Activity of Sulfate-Reducing Bacteria in Underground Waters of Rozdol

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In the account of our previous work on Rozdol from the materials of the 1958 expedition (Ivanov [1960b]) we investigated the distribution and activity of sulfate-reducing bacteria in the waters of the Upper Tortonian water-bearing horizon of the Rozdol field. However, experiments on the Yazov field showed that in the investigation of hydrogen sulfide formation in the Carpathian deposits, it is essential to take into account the activity of sulfate-reducing bacteria in other water-bearing horizons too, particularly in the waters of the Lower Tortonian horizon underlying the gypsum-anhydrite layer (Ivanov and Kostruba [1961]).

Hence, the first aim of the present work was to study the distribution of sulfate-reducing bacteria not only in the waters of the Upper Tortonian limestone horizon, but also in the waters of the gypsum-anhydrite layer and in the Lower Tortonian water-bearing horizon.

A second aim of the work, also suggested by investigations on Yazov, was to study the seasonal changes in the rate of sulfate reduction by means of short-term experiments involving the use of isotopes (Ivanov [1960a]).

Finally, the last aim of the work was an experimental investigation of the activity of sulfate-reducing bacteria in long-term experiments with samples of underground waters. The material for the work was collected in two expeditions in March-April and in June-August, 1959.

The results of a study of the distribution of sulfate-reducing bacteria in waters of the Rozdol field are given in Fig. 1. The study of the occurrence of these bacteria in waters of the limestone water-bearing horizon completely confirmed our earlier data: Sulfate-reducing bacteria were found in every sample of water both from the region of sulfate-calcium hydrogen sulfide waters (all the drainage wells and wells 150 G, 37 G, and 31 G) and from the zone of fresh hydrocarbonate-calcium waters, which do not contain hydrogen sulfide (wells 7G, 9 G, 11 G). It should be noted that in carrying out the

present work we did not take the samples from the observation wells by means of a bailer, as was done in 1958, but by a specially designed sampler with removable sterilized evacuated glass containers. Thus, in the 1959 investigations there was no possibility of the samples taken from H_2S -free wells being contaminated by microflora from the bailer.

The number of bacteria in samples from the drainage wells was usually low: usually growth was observed only when a 5 ml or 1 ml inoculum of underground water was used (Fig. 1). A quantitative estimate of the bacteria and the determination of the hydrogen sulfide content of waters of the observation wells were often impossible owing to the presence in the water samples of iron sulfide formed by the hydrogen sulfide corrosion of the casing pipes of the wells. Hence, quantitative data on the occurrence of sulfate-reducing bacteria in waters of the observation wells are not given, and the hydrogen sulfide content is indicated only for those samples which did not contain an admixture of iron sulfide (Fig. 1).

In addition to the samples of water from the limestone horizon we analyzed three samples of water from observation wells on the gypsum-anhydrite (wells 41G, 42G, 43G) and four samples of water from the Lower Tortonian sandstones (wells 32G, 36G, 33G, 38G - Fig. 1). In all these samples, apart from that from well 38G, we found cells of sulfate-reducing bacteria which gave a growth in Shturm's modified Tauson medium.

The obtained data on the numbers of sulfate-reducing bacteria and hydrogen sulfide content in waters of the gypsum and sandstone horizon (Fig. 1) confirm the view of hydrogeologists that these water-bearing horizons are hydraulically connected with the main water-bearing horizon of the Upper Tortonian limestones. However, the low hydrogen sulfide concentrations and the fairly high rH_2 in the waters of the underlying rocks (Fig. 1) would appear to indicate that the production of H_2S in

these waters is low, and that the bulk of the hydrogen sulfide in the Rozdol field is formed in the waters of the Upper Tortonian sulfur-bearing limestones.

The results of a study of the rate of sulfate reduction in the waters of the limestone horizon are given in the table. Comparing the values for the rate of sulfate reduction obtained in the spring series of experiments with the summer data, we see that in the summer period the rate of the process in the waters of almost all the wells was four to ten, or more, times greater than the rate of this process in the same waters in spring. An increase in the rate of hydrogen sulfide formation in summer was also indicated by the data on the variation of the hydrogen sulfide content of Rozdol waters (Fig. 2). These data reveal that the average hydrogen sulfide

content of the waters fell by more than 15 mg/liter during the period from July, 1958, to April, 1959. By the end of May the hydrogen sulfide content had fallen still further, but subsequently the fall was checked and right up to the end of November the hydrogen sulfide content of the water was practically constant.

The hydrogen sulfide content of the investigated waters was affected mainly by two processes: on one hand, the removal of hydrogen sulfide waters by the drainage wells and dilution of the hydrogen sulfide waters in the seam by H_2S -free waters collected from the supply region and, on the other hand, the continuous process of formation of hydrogen sulfide from sulfates in the waters. If we take into account the fact that during the whole period of observations from July, 1958,

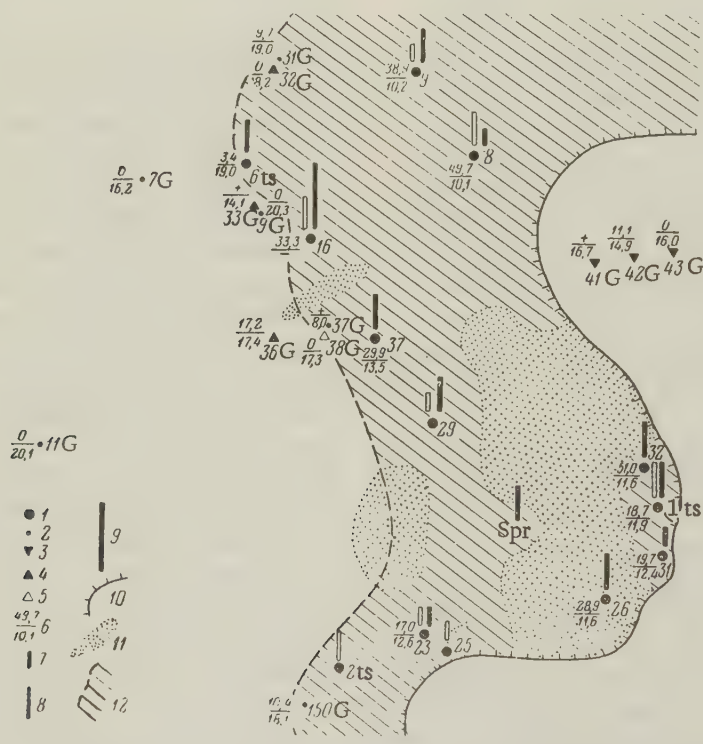


Fig. 1. Distribution of sulfate-reducing bacteria and hydrogen sulfide and variations in oxidation-reduction potential (rH_2) in underground waters of different water-bearing horizons of Rozdol sulfur field (1959). Wells in which sulfate-reducing bacteria were found: 1) Drainage wells; 2) observation wells on water-bearing horizon of Upper Tortonian limestones; 3) observation wells on gypsum-anhydrite layer; 4) observation wells on Lower Tortonian sandstone; 5) observation wells on sandstone in which no sulfate-reducing bacteria were found; 6) fraction indicates hydrogen sulfide content of well water (numerator) and rH_2 (denominator). Numbers of sulfate-reducing bacteria found in waters of drainage wells: 7) 200 cells per liter of water; 8) 1000 cells per liter of water; 9) 10,000 cells per liter of water. White columns give results of spring series of experiments, black columns give results of summer series. 10) Boundary of limestone water-bearing horizon; 11) regions of Upper Tortonian limestones dried out to bottom; 12) area of occurrence of hydrogen sulfide waters in water-bearing horizon of Upper Tortonian limestones.

Variations in Rate of Sulfate Reduction, Hydrogen Sulfide Content and rH_2 in Waters of Limestone Horizon of Rozdol Field (1959)

Well No.	March-April, 1959			July, 1959		
	H_2S , mg/liter	rH_2	sulfate reduction, mg H_2S /liter/day	H_2S , mg/liter	rH_2	sulfate reduction, mg H_2S /liter/day
9	37.4	11.3	0.089	38.9	10.2	0.579
8	49.3	11.6	0.101	49.7	10.1	0.017
16	36.7	12.3	0.069	33.3	—	0.270
23	16.3	15.5	0.002	17.0	12.6	0.022
1ts	37.4	12.4	0.015	18.7	11.9	0.207
31	30.9	11.6	0.009	19.7	12.4	1.675
32	51.3	10.3	0.031	51.0	11.6	3.232
5	18.9	11.0	0.045	—	—	—
29	20.3	19.0	0.021	—	—	—
25	27.7	14.5	0.003	—	—	—
37	—	—	—	29.9	13.5	0.024
26	—	—	—	28.9	11.6	0.073
Spring in face	—	—	—	38.0	—	0.056
2ts	50.1	12.9	0.007	—	—	—

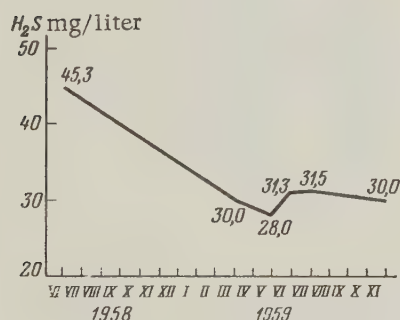


Fig. 2. Variation of hydrogen sulfide content of waters of limestone horizon of the Rozdol sulfur field. Mean figures for all operating drainage wells.

through November, 1959, the monthly quantities of H_2S -free water entering the seam and diluting the hydrogen sulfide water were approximately the same, it becomes obvious that the stoppage in the fall of the hydrogen sulfide content of the Rozdol waters in the summer months can be attributed entirely to an intensification of sulfate reduction.

Thus, direct investigation of the rate of sulfate reduction by the labeled atom method and a study of the hydrogen sulfide balance in the extracted waters indicate seasonal changes in the rate of hydrogen sulfide formation in the Rozdol sulfur field. We recall that we obtained similar results in a study of the Yazov deposit (Ivanov and Kostruba [1961]).

The aim of our work was also to elucidate the conditions for the activity of sulfate-reducing bacteria in

Rozdol waters. As we noted earlier, the concentration of sulfates and the low oxidation-reduction potential in waters of Rozdol and other Carpathian sulfur deposits are quite congenial for the development of sulfate-reducing bacteria (Ivanov [1960a, b]; Ivanov and Kostruba [1961]).

However, the presence of organic matter and certain biogenic elements, primarily nitrogen and phosphorus, is still an essential factor for the high activity of sulfate-reducing bacteria.

The 1958 experiments in which the BOD method was used to reveal the presence of organic matter assimilable by microorganisms gave positive results, but these experiments were conducted with water samples which were taken outside the region of occurrence of hydrogen sulfide sulfate-calcium waters (Ivanov [1960b]) and which were not typical of the Rozdol field. We corrected this fault during the course of the present work by making determinations of the BOD in water samples collected from the region of sulfate-calcium waters typical for the field. The hydrogen sulfide was removed from these samples by aeration. The results of these experiments, which are given in Fig. 3, show that in these waters the biochemical oxygen consumption for oxidation of the dissolved organic matter was very vigorous and in several samples (wells 8, 1ts, 37) 80 to 100% of the dissolved oxygen was consumed within two days.

Another method employed for a study of the supply of dissolved organic matter suitable for sulfate reduction was prolonged incubation of flasks containing underground water and natural microflora in laboratory conditions. As the data given in Fig. 4 show, the estimated increase of hydrogen sulfide in this series of experiments varied from 1.25 to 11.5 mg H_2S per liter in 20 days. It

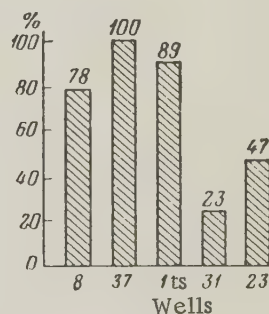


Fig. 3. Values of biochemical oxygen consumption in sulfate-calcium waters of Rozdol sulfur field. Columns indicate percentage of oxygen consumed in two days after start of experiment.

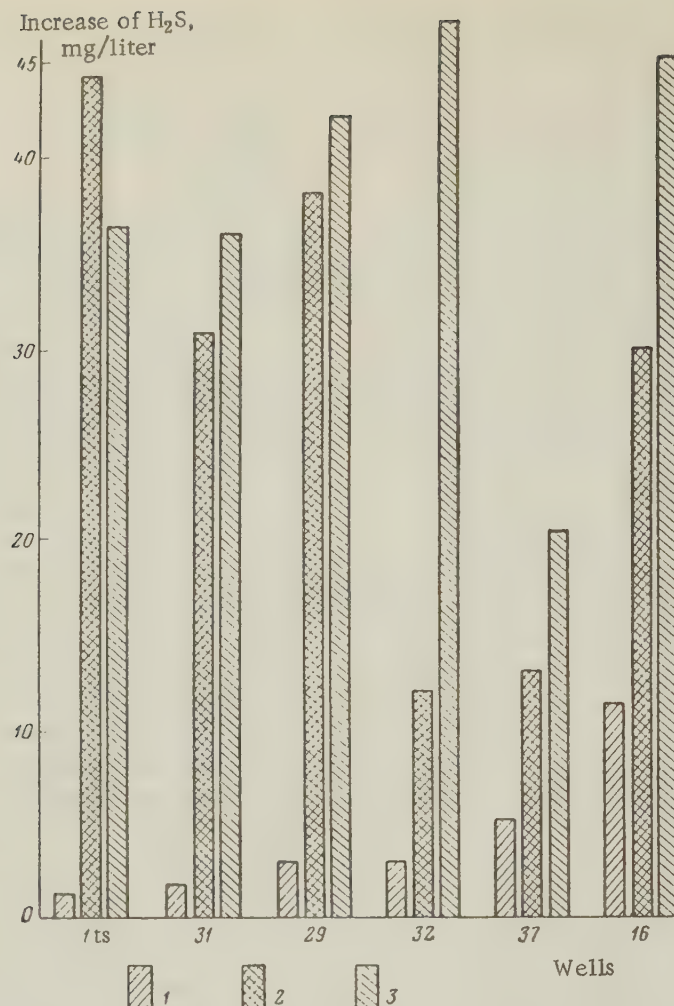


Fig. 4. Hydrogen sulfide production in samples of underground waters from Rozdol sulfur field. Twelve-day incubation of sample in laboratory. 1) Water with no additions; 2) water with addition of 1 g of calcium lactate per liter; 3) water with addition of calcium lactate and salts of phosphorus and nitrogen.

should be noted that, owing to the poor quality of the ground connections of the flasks and several other faults due to the experimental procedure in the field, there was some loss of hydrogen sulfide in the experiments. Thus, the actual increase of hydrogen sulfide resulting from the utilization of organic substances and sulfates in the water was greater than the increase estimated in the experimental flasks. Hence, we will not examine the increase in hydrogen sulfide in each individual well, but will only note that the obtained data definitely show that all the conditions required for microbiological reduction of sulfates were present in the investigated waters.

For a fuller analysis of the effect of individual factors on the activity of sulfate-reducing bacteria in Rozdol waters we conducted other experiments in parallel with those involving incubation of underground water

containing no additions. In these other experiments we incubated the same water with the addition either of mineral salts of nitrogen and phosphorus in concentrations of 100 mg N/liter (in form of NH_4Cl) and 100 mg P/liter (in form of K_2HPO_4), or of organic matter (1 g of calcium lactate per liter), or of a combination of mineral salts and calcium lactate. The conducted experiments showed that the addition of only the mineral components of the nutrition of sulfate-reducing bacteria did not lead to a stimulation of hydrogen sulfide production (Fig. 4). On the other hand, the addition of organic matter greatly stimulated sulfate reduction — the estimated increase of hydrogen sulfide in 20 days frequently amounted to 30–40 mg/liter, i.e., it was several times greater than the increase of hydrogen sulfide when no additions were made (Fig. 4). An even greater stimulation of sul-

fate reduction was produced by the "feeding" of sulfate-reducing bacteria with a combination of organic matter and mineral salts.

The data of these experiments show that the main factor limiting the activity of sulfate-reducing bacteria in Rozdol underground waters is the lack of organic matter, since the addition of organic matter causes a stimulation of hydrogen sulfide production.

SUMMARY

1. Sulfate-reducing bacteria occur in all three hydraulically connected water-bearing horizons of the Rozdol sulfur field, but the horizon producing most hydrogen sulfide is the water-bearing horizon of the Upper Tortonian limestones.

2. The rate of sulfate reduction in underground waters in the summer months greatly exceeds the rate of this process in spring.

3. Prolonged experiments with samples of underground waters of the Rozdol deposit showed that all the conditions required for the microbiological reduction of sulfates are present in these waters.

4. The amount of organic matter dissolved in the water is the main factor limiting the development of sulfate-reducing bacteria and the production of hydrogen sulfide from sulfates by these bacteria.

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MICROZONAL OCCURRENCE OF OXIDATIVE PROCESSES IN SULFUR ORE OF THE ROZDOL DEPOSIT

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As the studies of Ivanov et al. [1958] showed, Thiobacillus thiooxidans takes an active part in the sulfuric acid weathering of sulfur deposits in Shorsu, Gaurdak and Vodino.

A study of the role of T. thiooxidans in the sulfuric acid weathering of Rozdol sulfur deposit (Karavaiko [1959]) showed that this microorganism was practically absent in the sulfur-bearing stratum itself.

For instance, in sulfur-bearing limestones covered by Tertiary Sarmatian and Tortonian deposits T. thiooxidans could not be found at all. This microorganism was found only in those parts of the field where the sulfur-bearing stratum emerged directly under Quaternary permeable deposits. Thus, we could not say with certainty that oxidative processes were of very widespread occurrence in the sulfur-bearing limestone of the Rozdol deposit. Further evidence of the existence of oxidative processes in the deposit would be the discovery of acidification of the ore due to the formation of free sulfuric acid. However, in none of the bulk samples from any locality could we discover a fall in pH, which was either neutral or alkaline. An assessment of sulfuric acid production from the increase in sulfates was also impossible owing to the presence of gypsum in the limestone deposits.

This compelled us to make a different approach to the question of the occurrence of oxidative processes in the field, viz., to try to devise a more sensitive micro-method which would enable us to discover foci where sulfur was oxidized to sulfuric acid. This was suggested to us by the many known published facts, particularly the fact that the result of the activity of microorganisms is more likely to be manifested where the most favorable physicochemical conditions for their activity are created. These conditions may be very distinctive and may occur within the narrow confines of microzones (Perfil'ev [1926]).

The numerous classical methods are unsuitable for investigation of the physicochemical conditions in microzones. Such an investigation, as Perfil'ev points out, requires sensitive micromethods which will permit a determination of the active acidity in a very small volume of the initial sample.

The employment of micromethods enabled him to demonstrate the important role of microconditions in the distribution of microorganisms in mud deposits in waters and in many processes such as the formation of lacustrine manganese iron ores.

In our work we decided to approach the study of the oxidative processes in the field with due regard to microconditions, both in the taking of the samples and in their analysis. The main aim of our work was to establish the occurrence of the oxidation of sulfur to H_2SO_4 in the sulfur-bearing stratum of the Rozdol field with reference to the distribution of T. thiooxidans.

METHOD

To study the process of sulfur oxidation in the deposit itself we employed an indicator paper micromethod which we had devised for the determination of active acidity.

In this method small pieces of ground specimens of sulfur ore are laid out on damp indicator paper and left till the latter dries out.

The indicator paper was prepared in the following way. Pieces of filter paper were alkalized to neutral pH with 0.01 N NaOH solution. They were then immersed for 5 min in a solution of a suitable indicator in a Petri dish. As indicators we used methyl red, which changes from red to yellow in the pH range 4.4-6.0, and thymol blue, which changes from red to yellow in the pH range 1.2-2.8. If acidification microzones were present, red spots appeared on the paper.

At the same time we made determinations of the active acidity in the bulk sample by means of the potentiometer P-6 and counted the numbers of T. thiooxidans by the method described in a previous paper (Karavaiko [1959]).

RESULTS

Study of oxidative processes and distribution of *Thiobacillus thiooxidans* in sulfur-bearing limestone of Rozdol sulfur field.

The work was conducted in a longitudinal trench of this field. The sulfur-bearing limestones here were heavily fissured and karstic. The fissures were filled with clayey particles and disintegrated rock.

No. of sample	Description of sample	pH from bulk analysis of rock sample	pH in microzones	Number of <i>T. thiooxidans</i> cells in 1 g of sample
1	Scraping from fissure in sulfur-bearing limestone (bench I of longitudinal trench)	8.0	-	10-100
2	Crumbling sulfur-bearing limestone in niche (bench I of longitudinal trench)	7.50	4.0	10,000
3	Rain water gathering at foot of trench wall	7.60	and less Not determined	100
4	Ferruginous coating in niche (bench II of longitudinal trench)	7.40	3.0	10,000
5	Very crumbly blocks of sulfur-bearing limestone	7.5-8.0	4.4	10-10,000
6	Ferruginous clay in fissures (bench II of longitudinal trench)	7.50	4.0	10,000
7	Sulfur-free piece of limestone from sulfur-bearing block	7.9	-	0
8	Finely divided sulfur on surface of clay	7.75	3.5	10,000
9	Scraping from surface of broken-off block of sulfur-bearing limestone	7.65	-	1000

The samples for the investigation were taken in the heavily fissured walls of the longitudinal trench and from weathered blocks of sulfur-bearing limestone.

The table reveals microzones with pH below 4.0 were present in disintegrating sulfur-bearing limestone. We succeeded in obtaining imprints of these microzones in the form of red spots on the indicator paper from samples 2, 4, 5, 6 etc. (see table). It can be seen from the table that these samples came mainly from fissures and niches. It is particularly important to note that it was in these samples that large numbers of *T. thiooxidans* were found. The number of these bacteria in such samples reached 10,000 cells per g (see table), whereas in samples where no microzones with low pH could be found, the number of bacteria varied from 10 to 100 cells per g of sample, or were absent altogether.

Thus, we can now conclude that oxidation of sulfur to sulfuric acid is taking place in the sulfur-bearing limestone of the Rozdol field. The evidence for this is the very low pH in the microzones.

The presence of large numbers of *T. thiooxidans* in the oxidation zones indicates the biogenic nature of the oxidative processes in the deposit.

A potentiometric determination of the active acidity did not enable us to detect the small quantities of sulfuric acid accumulating in free form in the microzones. Hence, from bulk analyses of the active acidity we could not determine the presence of sulfur oxidation processes in the Rozdol field.

The microzonality of the oxidative processes indicates that these processes have still not developed to the

extent that the sulfuric acid formed can destroy the sulfur-containing carbonate rock and acidify the environment. This is presumably due to two factors: 1) the sulfur-bearing stratum has been exposed for a relatively short period so far (about two years); 2) in carbonate rocks, unfavorable conditions for the development of *T. thiooxidans* are created owing to the alkaline reaction of the containing limestones and the low humidity.

Hence, *T. thiooxidans* preferentially colonizes the niches and fissures, where the humidity is high, and forms oxidation microzones.

The author regards it as a pleasant duty to express his thanks to S. I. Kuznetsov and M. V. Ivanov for guidance in this work.

SUMMARY

1. In Rozdol sulfur field oxidation of sulfur ore leading to the formation of sulfuric acid begins in microzones where the sulfur is presumably in the most finely divided state.

2. An analysis showed high numbers of *Thiobacillus thiooxidans* in acidified microzones, thereby suggesting an important role for this organism in sulfur oxidation in the ore of the Rozdol field

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ROLE OF CHEMOSYNTHESIS IN THE PRODUCTION OF ORGANIC MATTER IN RESERVOIRS

V. Photosynthesis and Chemosynthesis in the Bays of Kuibyshev Reservoir

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It was shown in previous studies (Sorokin [1957, 1958]) that in a number of cases chemosynthesis may play an important role in trophic relations in waters. The source of energy for the development of chemosynthetic microbes in water is the oxidation of the end products of the anaerobic decomposition of organic matter in mud deposits. Thus, chemosynthesis is a process succeeding photosynthesis, since in the final analysis chemosynthesis entails the use of energy bound during photosynthesis (Sorokin [1960a]). In the light of these remarks it was of interest to study the relative values of the daily production of chemosynthesis and photosynthesis when the rate of the latter is high and the conditions for the development of chemosynthetic microbes are most favorable.

In view of these considerations we made determinations of the rate of photosynthesis and chemosynthesis in Cheremshan and Usa Bays of Kuibyshev Reservoir in summer, 1957. The time when this work was conducted coincided with the filling of Kuibyshev Reservoir to the projected level. During the high water of 1957 large areas of fertile flood plain soils were inundated. Leaching of biogenic elements from the soil caused a sudden bloom of phytoplankton in the reservoir. The primary production of phytoplankton photosynthesis was particularly high in the bays. For instance, in Cheremshan Bay the production throughout the growing period was 670 g/m^2 , expressed as glucose (Salmanov [1960]). Owing to the existence of the distinct stratification resulting from the calm weather in July-August, 1957, an anaerobic zone was created in the bottom layer of water in the bays.

It was shown in previous investigations that the most favorable conditions for the development of chemosynthetic microbes are created at the junction of anaerobic and aerobic zones and that here the rate of chemosynthesis reaches a maximum (Lyalikova [1957]; Sorokin [1957]). Thus, in the bays of Kuibyshev Reservoir we had the opportunity to determine the relative rates of

photosynthesis and chemosynthesis in optimal natural conditions for their occurrence.

Besides the analyses of photo- and chemosynthesis we also studied the vertical distribution of the planktonic water fleas *Daphnia longispina*, which are fine filterers and feed largely on bacteria (Rodina [1950]; Manuilova [1954]). Our observations during the winter showed that the vertical distribution of the *Daphnia* population was closely correlated with the distribution of chemosynthesis in the water. This indicates the important trophic role of chemosynthesis (Sorokin [1957]).

Hence, it was of interest to examine the relation between the rate of chemosynthesis and the numbers of *Daphnia* at different depths in summer, when the water is stratified and there is an abundant development of phytoplankton (*Aphanizomenon*, *Microcystis*, and *Anabaena*) of little use as food for water fleas. Below we give the results of these determinations.

METHODS AND RESULTS

The primary production of organic matter by photosynthesis and the production of chemosynthesis were determined by the use of the carbon radioisotope C^{14} . The method of these determinations has been fully described in previous papers (Sorokin [1957, 1958]). In calculations of the production values we introduced a correction, equal to 6%, for the isotope effect (Sorokin [1960b]).

The gases methane and hydrogen dissolved in the water were extracted by boiling a sample of about 3 liters. The gases were extracted in the ship's laboratory immediately after the samples were collected.

The results of measurements of the daily values of photosynthesis and chemosynthesis, and of the dissolved oxygen content and numbers of filtering crustacea at different depths in the water, are shown in Figs. 1-5. As can be seen, the layer in which photosynthesis occurred did not exceed 3 m owing to the relatively deep color of the water and the low transparency (0.8-1.2 m, measured by disk). Nevertheless, the phytoplankton devel-

opment was so intense that the production of photosynthesis was 3 to 8 g of carbon (or 7.7-21.0 mg O_2 per liter) per day under 1 sq m of water surface. The settling of the mass of dying algae on the bottom and their accumulation owing to the calm weather conditions and the lack of current in Cheremshan and Usa Bays produced an oxygen deficiency in the bottom layer. In

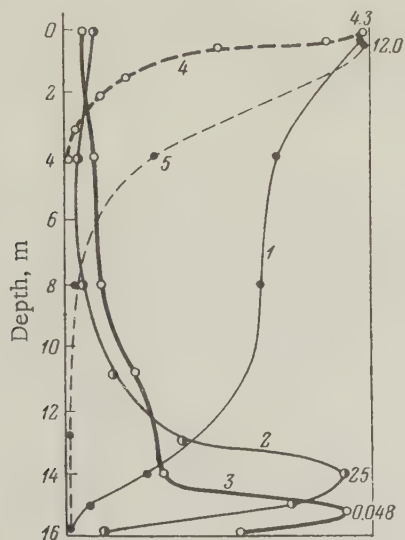


Fig. 1. Results of analyses at different depths; bed of R. Usa 8 km from mouth, 1) O_2 , mg/liter; 2) *Daphnia*, thous/ m^3 ; 3) chemosynthesis, mg C/liter; 4) photosynthesis, mg C/liter; 5) phytoplankton (relative value).

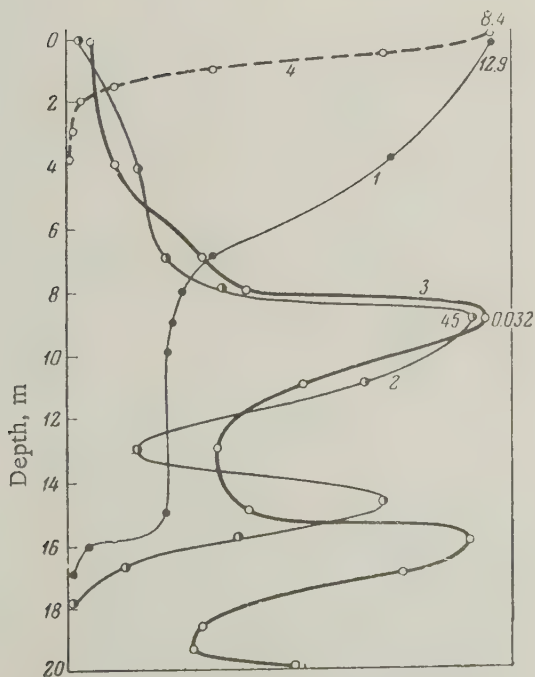


Fig. 2. Same as Fig. 1, bed of R. Cheremshan 15 km from mouth. Designations as in Fig. 1.

Usa Bay a complete absence of oxygen was recorded only at the bottom (Fig. 1). Over the Cheremshan River bed (Figs. 2-3) and over the bed of a terrace stream (Fig. 4) the thickness of the anaerobic zone was about 3 m, and the appearance of free hydrogen sulfide was observed in this zone. On the flood plain parts of Cheremshan Bay an anaerobic zone 1.5-2 m thick was also formed (Fig. 5). The decay of the phytoplankton and remains of inundated vegetation in anaerobic conditions was accompanied by the development of the sulfate-reduction process with the formation of hydrogen sulfide (Kravtsov and Sorokin [1959]) and evolution of gases. Analyses of the gases extracted from water samples collected from the bottom layer on the Cheremshan River bed showed that this water contained 3.8 cm^3 of dissolved methane and 0.4 cm^3 of dissolved hydrogen per liter. Over the Usa bed and over the Cheremshan flood plain the content of dissolved methane and hydrogen lay between 0.3 and 0.4 cm^3 /liter.

An analysis of the daily values of chemosynthesis at different depths showed that at the boundary between the aerobic and anaerobic zones, in the layer where the oxygen content fell most rapidly, there was an intense production of organic matter owing to the activity of chemosynthetic microbes. The daily production of chemosynthesis per liter in this layer was 0.025 to 0.056 mg of organic carbon in the form of bacterial cells. The increase in bacterial population corresponding to these values of chemosynthesis was 0.5 to 1 mill cells per 1 ml, the general bacterial content of the water in the reservoir being about 2 mill/ml (Salmanov [1959]). Thus, in the layer where the fall in O_2 content is steepest, there is an intense new production of bacterial protein by chemosynthetic bacteria, which are assimilated well by Cladocera (Sorokin [1959]). This explains the regularly

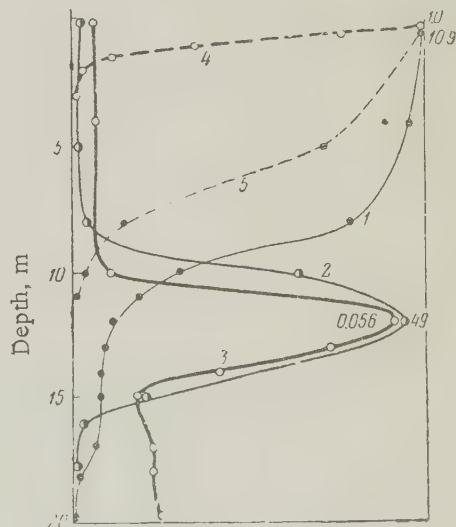


Fig. 3. Same as Fig. 1, bed of R. Cheremshan 20 km from mouth.

noted phenomenon of concentrations of *Daphnia* in the layer where chemosynthesis is most intense. The graphs reveal that *Daphnia* do not congregate in the zone of intense photosynthesis and high phytoplankton biomass, but in those layers where the high rate of chemosynthesis results in the new production of particles of formed food consisting of bacterial bodies and at the same time oxygen is present in sufficient quantity (not less than 1 mg/liter).

In Usa Bay above the bed of the Usa River (Fig. 1), a complete absence of oxygen was noted only in the very bottom layer. The maximum daily chemosynthesis (0.048 mg C/liter) was recorded here at depth 15.3 m, and the maximal concentration of *Daphnia* at depth 14 m, the total depth being 16 m. In the very bottom layer, where oxygen was completely absent, the rate of chemosynthesis was appreciably lower. In the surface layer at all stations the production of chemosynthesis was low and did not exceed 0.003 mg C/liter per day.

At stations located on the Cheremshan River bed (Fig. 2) and on a terrace stream (Fig. 4), we found two maxima for the rate of photosynthesis, which corresponded with the depths at which the fall in dissolved O_2 was steepest. It was of interest that at one of these stations two maxima for the rate of chemosynthesis (9 and 16 m) and two maxima of *Daphnia longispina* distribution (9 and 15 m) corresponded with the two steepest falls in O_2 content.

The high rate of chemosynthesis at the boundary of the anaerobic and aerobic zones is correlated with the occurrence there of oxidation of products of anaerobic decomposition, which accumulate in the anaerobic zone. Within the anaerobic zone itself chemosynthesis is suppressed by the lack of oxygen. Nevertheless, in the anaerobic zone chemosynthesis is much more intense than in the surface water. Chemosynthesis within the anaerobic zone is due to the activity of chemosynthetic anaerobes such as *Vibrio desulfuricans* or *Thiobacillus denitrificans*.

A determination of the values of the daily production of photosynthesis and chemosynthesis at different depths enabled us to calculate graphically the production under 1. m² of the water surface (Sorokin [1958]). The results of these calculations are given in the table. They show that in eutrophic water of medium depth (15-20 m), the daily production of chemosynthesis in the most favorable conditions (stratification and presence of anaerobic zone) amounts to 2.2 to 6% of the daily production of photosynthesis.

Despite the fact that chemosynthesis gives a relatively low production as compared with photosynthesis, its trophic role in the water is important. This is clear merely from the example of the above data on the concentration of zooplankton in the zone of intense chemosynthesis and the previously published data on the active feeding of various representatives of abundant forms of aquatic invertebrates on the autotrophic microflora de-

veloping in natural water (Lebedeva [1959]; Sorokin [1960a]). In assessing the trophic role of chemosynthesis we must take into account that this process is localized at the boundary of the aerobic and anaerobic zones. At the junction of these zones the production of chemosynthesis reaches considerable values. These values sometimes approximate to the values of the daily production of photosynthesis at the surface. The consumers of the production of chemosynthesis — the invertebrates — are capable of active migration in search of food and they congregate at depths where new production of organic matter is taking place. The organic matter formed by

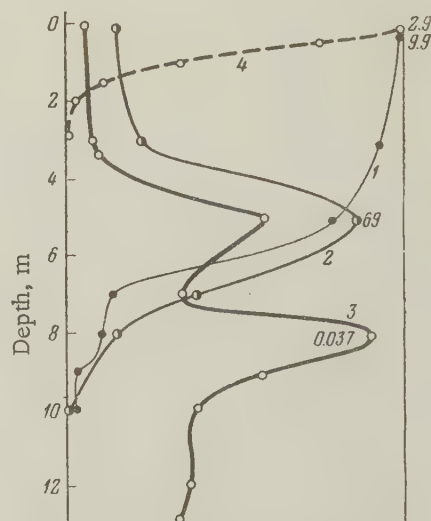


Fig. 4. Same as Fig. 1, bed of terrace stream in Cheremshan Bay.

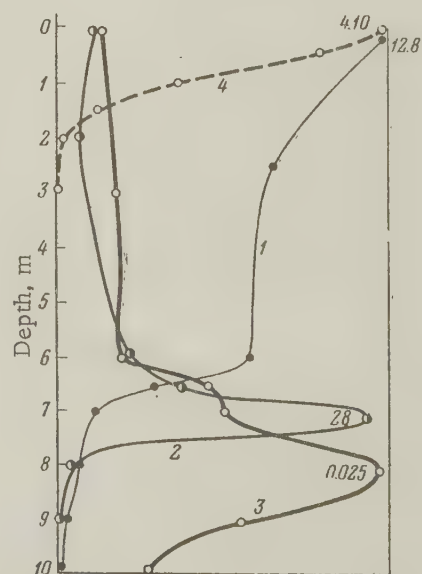


Fig. 5. Same as Fig. 1, left bank flood plain of R. Cheremshan 20 km from mouth.

Relation between Production of Photosynthesis and Chemosynthesis per m² of Water Surface

No. of station	Photosynthesis, g C/m ² per day	Chemosynthesis, g C/m ² per day	Production of chemosynthesis as percentage of photosynthesis
35	3.61	0.169	4.70
28	5.30	0.278	5.25
30	7.70	0.275	3.57
4	3.76	0.086	2.28
3	2.69	0.189	7.05

chemosynthesis is excellent food for aquatic invertebrates, since it consists of particles of animal protein. The latter fact is of particular importance in a consideration of the role of chemosynthesis in the trophic interrelationships of organisms in Volga reservoirs. The fact is that in Volga reservoirs there develop large phytoplankton forms which are not easily assimilated by cladocerans. Hence, in such reservoirs the process of biological production is accomplished with the participation of bacteria as an intermediate link (algae → bacteria → zooplankton). In these conditions the importance of chemosynthesis as a process leading to the accumulation of a supply of bacterial food for zooplankton is certainly enhanced.

SUMMARY

1. A study was made of the relative production of photosynthesis and chemosynthesis in Cheremshan and Usa Bays of Kuibyshev Reservoir in the first year of its filling to the planned level.

2. It was found that with high primary production of photosynthesis and with optimal conditions for the development of chemosynthetic bacteria (stratification and presence of oxygenless zone in bottom layer), the daily production of chemosynthesis per m² of reservoir surface was 3-7% of the daily production of photosynthesis.

3. The greatest values of chemosynthesis were recorded at the boundary of the aerobic and anaerobic zones, where the fall in dissolved oxygen content was steepest.

4. Concentrations of *Daphnia* were noted at the depths of maximal chemosynthesis. This indicates that chemosynthesis plays a definite part in the production of bacterial food for aquatic invertebrates.

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MICROBES OXIDIZING THIOCYANATE AND CYANIDE COMPOUNDS IN WASTE WATERS OF COKE BY-PRODUCT PLANTS

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The aim of the present paper is to give a characterization of the thiocyanate-decomposing microbes which we have cultured, to describe the method of culturing them and our observations of the changes in microbial saprophytes of a biological film and activated sludge when they were cultivated in synthetic media with ammonium thiocyanate as the only source of nitrogen and carbon.

In 1952 we undertook the cultivation, isolation and selection of the most active thiocyanate-decomposing microbes so that we could use these microbes in conjunction with phenol-decomposing microbes for purification of phenol waste waters from coke by-product plants. At that time phenol-decomposing microbes had already been successfully introduced into the biochemical installations of Kadiievka and Kharkov coke by-product plants by Giprokoks (State Institute for Planning of the Coke By-Product Industry) in collaboration with the Ukrainian Institute of Communal Hygiene and the staffs of the plants. The use of phenol-decomposing microbes alone reduced the harmful effect of phenol waste waters on certain rivers (Uda and others) so much that in these rivers and on their banks normal plant and animal life survived. However, the indices of the dephenolized waters still did not correspond with the requirements demanded of purified water.

The successive employment of phenol-decomposing and thiocyanate-decomposing microbes (first stage of purification) made it possible to effect a high degree of purification of phenol waste waters (in such waters nitrification processes occurred). After the first stage of purification the phenol waste waters were completely purified by subsequent passage through a biofilter (Putilina [1952, 1956, 1959a, b]). The complete purification of phenol waste waters was carried out in an experimental biochemical unit of the industrial type in the Ukrainian Research Institute of Communal Hygiene (N. T. Putilina, G. S. Gan, E. M. Yurovskaya, and others).

Giprokoks is at present collaborating with the Kadiievka coke by-product plant in a test of this two-stage method on an industrial pilot unit. The results obtained

so far confirm that complete purification of phenol waste waters can be accomplished by the use of this method.

Abson and Todhunter [1959] suggested a method of purification similar to that employed by us.

Happold and Key in 1937 first isolated from gas plant waste waters a microbe capable of multiplying only in solutions of sodium thiocyanate or ammonium thiocyanate in phosphate buffer. As a result of the vital activity of this microbe ammonium sulfate and carbon dioxide were formed in the medium. This microbe grew in the form of a dewdrop colony on the surface of ordinary nutrient agar. After growth on nutrient agar the microbe lost its ability to decompose thiocyanate compounds even in inorganic solutions. On meat-peptone agar the colonies were a pale yellowish-green color. These authors isolated pure cultures from a mixed colony of three microbes. One of them oxidized thiocyanate compounds in media containing inorganic salts (0.01 M NaCNS solution and 0.01 M phosphate buffer solution at pH = 6.9). The presence of organic substances limited the growth of this microbe. They called it Bacterium thiocyanooxidans.

For cultivating the thiocyanate-decomposing microbes we used the method which we employed for phenol-decomposing microbes (Putilina [1959a]).

As a selective nutrient medium for this purpose we chose a synthetic medium No. 1 of the following composition: tap water 1 liter, ammonium thiocyanate 0.2-0.4 g, mono- and disubstituted potassium phosphate 0.5 g each, magnesium sulfate 0.1-0.2 g, and traces of ferrous sulfate. Hundred ml samples of medium No. 1 were put into 30 vials of 200 ml capacity. These vials were divided into three groups - series. We put 1 ml of biological film into each of the first series of vials, and 1 ml of activated sludge into the second series. The film and mud were obtained from Kharkov Town Biological Station. The vials of the third series were inoculated with 1 g of soil taken in outflows of industrial waste waters from a coke by-product plant. The experimental samples were incubated at 25-30 C until the experimental liquid ceas-

ed to give a positive reaction for thiocyanates. One vial from each experimental series was opened for investigation and only when there was a change in the picture of the microscopic smears prepared from the experimental samples did we open the second vial. The results of the analyses were collated. With further changes in the experimental samples we opened the third vial, and so on.

The duration of incubation of the experimental vials depended on the source of the inoculum. In experimental samples of the first series (containing film) an appreciable reduction of thiocyanate concentration was noted after 25 days. The second series (containing sludge) gave the same result after 29 days, and the third (containing soil) after 13 days. The oxidation of ammonium thiocyanate was completed after 30-40 days in the first two series, and after 26 days in the third series. When the microbes had completely oxidized the ammonium thiocyanate we transferred approximately 10-20 ml of this liquid from an unopened vial into new sterile medium No. 1. Subculturing in medium No. 1 was continued over a period of half a year. In this way we obtained "natural complexes of thiocyanate-decomposing" microbes.

During the cultivation of the natural complexes of thiocyanate-decomposing microbes in defined medium No. 1, the composition of the microflora underwent marked changes. For instance, when smears of the experimental liquids from the first two series of experiments were examined 10-15 days after the start of the experiments the microscope fields were quite empty. We not only failed to discover normal microbes or protozoa but there were not even remains in the form of slimy clumps, etc. Such a picture was observed for almost two months. It must be noted that during this period, i.e., during the two months after the experiments had been set up, the ammonium thiocyanate in these flasks was oxidized fairly rapidly (within two days). We surmise that the microbes in the experimental liquids probably existed in an invisible form. We obtained evidence of this when these liquids were plated out on Petri dishes containing meat-peptone agar. On this medium there grew numerous very tiny dewdrop colonies, barely visible to the naked eye. There was no growth on agar medium No. 1.

In smears from the experimental samples of the first and second series after two months we discovered amorphous slimy clumps and large colorless spheres; in the slimy mass we occasionally found very tiny rod-like granules. It was not until the 70th to 72nd day that there began to appear in the smears, beside the granular slimy mass, very tiny ovate rods, very rare longer rods and large spheres, apparently burst. In the microscope field the rods were mostly lying freely (not in the slimy mass). On MPA at this time there grew the same dewdrop colonies as before. Seedings from these colonies in liquid thiocyanate medium did not cause oxidation of the thiocyanates.

Plating out on a solid thiocyanate medium after 2.5 months of subculturing produced a growth in the form of very tiny dewdrop colonies visible only under the low power of the microscope. Plating on MPA also produced tiny dewdrop colonies, but of slightly larger size than before, and also flat colonies with scalloped edges. The latter were few in number. As before, we found no oxidation of thiocyanates when liquid thiocyanate medium was seeded from the MPA.

We found similar changes in the microflora in the experimental samples inoculated with soil. In this case we did not manage to observe pictures of complete absence of microbes or their remains (empty fields under microscope). Otherwise the changes in the microflora in these experiments proceeded in the same sequence as in the experiments of the first and second series. The different stages gave way to one another much more rapidly.

As we already mentioned, subculturing in defined medium No. 1 was continued for half a year. By this time the ability of the microbes to oxidize thiocyanate compounds was consolidated. To train these microbes to decompose thiocyanates in phenol-free waste waters, i.e., in industrial conditions, we decided to make subcultures in medium No. 2, which was made up by adding 0.2 g of phosphates to 1 liter of dephenolized waste water (0.1 g each K_2HPO_4 and KH_2PO_4 per liter). Thiocyanates and nitrogenous compounds were already present in the waste water.

At this stage all the microbes from all the colonies used possessed slight motility.

As a result of this work we obtained 60 so-called natural complexes of thiocyanate-decomposing microbes. We selected the most active of these. From the selected complexes we attempted to isolate pure cultures on solid thiocyanate medium. However we did not manage this at this stage, since plating out of individual colonies produced neither growth nor oxidation of thiocyanates. Hence, we employed a coarse purification method at first: we inoculated liquid thiocyanate medium No. 2 with a large number of colonies taken up by a loop. With this method of seeding we obtained growth and decomposition of thiocyanates. After rearing such cultures and subcultures for two to three months we again plated them out on thiocyanate agar. This time we obtained larger dewdrop colonies, visible to the naked eye, and also a small number of still larger flat colonies with scalloped edges. This time we succeeded in obtaining growth in liquid thiocyanate medium and oxidation of thiocyanates by microbes isolated from a single dewdrop colony. These were very tiny short and stout rods, resembling the colon bacillus in shape, but only one-fifth to one-quarter as large. From the scalloped colonies there developed slightly longer rods with straight margins. Both cultures were slightly motile. By fivefold subculturing and isolation of microbes from the isolated

colony we obtained 60 pure cultures of thiocyanate-decomposing microbes. From this number we selected the ten most active cultures, which were tested for pathogenicity in the Mechnikov Institute of Vaccines and Sera in Kharkov.

Nine of these cultures—Nos. 1, 5, 17, 27, 27M, 42, 48 and 56 [only these eight enumerated in Russian original—Publisher]—were approved as nonpathogenic; culture No. 2 was pathogenic to mice.

The isolated microbes are capable of oxidizing up to 1000 mg of ammonium thiocyanate per liter in phenol-free waste waters. They also oxidize cyanide compounds in these effluents. The optimal temperature for their activity is 25-30°C, pH=6.8-7.2, and they oxidize thiocyanates well at pH=7.4-7.6. Their biochemical properties are now consolidated. All the microbes have become nonmotile, do not affect milk and sugars of the usual color series, do not produce indole or hydrogen sulfide, do not liquefy meat-peptone gelatin, stain negatively or a vague pinkish-blue with Gram stain; on agar medium No.2 they grow in the form of dewdrop colonies of diameter 0.2-0.5 mm; the same colonies grow on MPA but they are a little larger and the MPA in this case becomes a yellowish-green color. Now inoculations from MPA into liquid thiocyanate medium not only give growth, but also oxidation of thiocyanates, although a long period is required for this and the activity of the process is greatly reduced. After some subcultures the activity is restored. (At earlier stages all the cultures possessed motility; some cultures, Nos. 49 and 56, liquefied MPB, culture Nos. 5, 17, 27M fermented glucose and produced acid; culture No. 48 was Gram-positive. All the cultures lost their ability to oxidize thiocyanates after being plated out on MPA).

We assigned these cultures to the species Pseudomonas nonliquefaciens (according to Bergey). They appear to be similar to Bacterium thiocyanooxidans Happold.

Before the biochemical unit was set in operation, the aeration tank was heavily inoculated with the thiocyanate-decomposing microbes. The dephenolized waste water for purification required tens of millions to hundreds of millions of thiocyanate-decomposing microbes per ml. This was achieved by gradual filling of the aeration tank, as we described in our papers on dephenolization of waste waters by phenol-decomposing microbes (Putilina [1952-1956]). When the suggested conditions were maintained, this single inoculation was sufficient for continuous operation of the plant.

Thus, from our observations we can surmise with considerable assurance that the natural complexes of thiocyanate-decomposing microbes which we have cul-

tivated are derived from certain microorganisms which were previously incapable of oxidizing thiocyanates. When these microbes were introduced into synthetic medium No. 1, in which ammonium thiocyanate is the only source of nitrogen and carbon, the type of assimilation must have been altered, and this led to a change in the nature of the microbes themselves. The newly acquired properties became hereditarily consolidated and new forms of microbes appeared.

SUMMARY

1. Thiocyanate-decomposing microbes closely related to Pseudomonas nonliquefaciens and similar to Bacterium thiocyanooxidans have been isolated and grown in pure cultures.

2. These microbes are capable of completely oxidizing thiocyanate and cyanide compounds in phenol waste waters of coke by-product plants.

3. When biological film or activated sludge is introduced into liquid defined media containing ammonium thiocyanate as the only source of carbon and nitrogen, the microbes of the film and sludge undergo pronounced changes. Presumably these microbes pass through an invisible stage and stages in which they have the form of an amorphous slime and a granular slimy mass, and then they again assume the normal size and shape of visible microbes. Oxidation of ammonium thiocyanate begins at the stage of the invisible forms. Natural complexes of thiocyanate-decomposing bacteria were obtained and pure cultures were isolated from them.

4. The cultures of thiocyanate-decomposing microbes have been used successfully in conjunction with phenol-decomposing microbes for purification of phenol waste waters from coke by-product plants. This test was performed in the experimental biochemical unit of the Ukrainian Research Institute of Communal Hygiene.

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UTILIZATION OF NITROGENOUS PETROLEUM COMPOUNDS BY MICROORGANISMS UNDER ANAEROBIC CONDITIONS

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Investigations of connate waters from the Tuimazy petroleum deposits revealed that the connate waters contained microorganisms which are able to utilize the nitrogenous compounds generally found in crude oil [Bogdanova, 1958; Nametkin, 1955].

The purpose of the present laboratory experiments was to determine the ability of microorganisms found in connate waters to decompose petroleum under anaerobic conditions with petroleum as their only source of nitrogen.

The petroleum decomposition experiments included analysis for total nitrogen in the off gas and in the petroleum before and after the experiment. Petroleum (sp gr 0.85) mixed with sand was put into a bottle,

which was filled with connate water (about 50 ml). The bottle was then covered with a rubber stopper containing a vent tube filled with the same water. The free end of the tube was connected to a gas receiver which was filled with salt solution. The experiments were conducted at 33C for seven months. Altogether, six bottles were used; each bottle contained petroleum and one of the six connate waters tested.

Decomposition of petroleum with production of gas was observed in only the two bottles in which the connate waters contained bacteria capable of utilizing the nitrogenous petroleum compounds. Connate waters in the other bottles did not contain bacteria capable of utilizing the nitrogenous petroleum compounds.

Analysis of Petroleum and of the Gas Produced by Petroleum Decomposition under Anaerobic Conditions

Expt. No.	Connate water	Composition of petroleum (%)						Composition of petroleum decomp. gas (%)				
		before expt.			after expt.			CO ₂	O ₂	combustible gases	nitrogen	
		C	H	N	C	H	N				total	biogenic
13	Specific gravity 1.031. Well located in the petroleum bearing portion of the northeastern region of the Tuimazy structure. Sample taken from depth of 1580 m.	82.72	12.35	0.36	83.88	12.67	0.22	3.62	14.43	0	81.95	27.12
15	Specific gravity 1.032. Flowing well located in southwestern part of the Tuimazy structure. Depth 1600 m.	83.80	12.37	0.20	82.97	12.14	0.14	0.5	0	28.09	71.41	71.41

Note: The petroleum samples were analyzed in the bituminous products laboratory of the All-Union Scientific Research Institute of Petroleum Geology Exploration. The gas samples were analyzed on the All-Union Heat Engineering Institute apparatus in the gas laboratory of the aforementioned Institute. The connate waters and petroleum studied did not contain NO₂, NO₃, or NH₃.

Final analysis of both petroleum samples showed some decrease in total nitrogen content; biogenous nitrogen was found in the off gas (table). Unfortunately, the quantity of off gas produced was insufficient for determination of the argon content; therefore, the quantity of biogenous nitrogen was estimated from the oxygen content.

The fact that deep connate waters contain bacteria which are able to utilize complex nitrogenous compounds in petroleum indicates that production of molecular nitrogen from nitrogenous compounds occurs in the depths.

These hypotheses confirm the opinion of some scientists concerning the relation of nitrogen gas accumulation to petroleum deposits (Sukhankin, 1947).

SUMMARY

Microorganisms found in connate waters are able to use nitrogenous petroleum compounds as a nitrogen source, and decompose petroleum under anaerobic conditions with the production of molecular nitrogen.

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BACILLUS SPECIES IN THE FISH-BREEDING PONDS OF LEDNIZE

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The presence of species of the genus *Bacillus* in surface waters has not, to date, been adequately studied; nevertheless the biological activity of these microorganisms has a significant influence on processes that occur in ponds. Members of the genus *Bacillus* play a large part, for example, in the decomposition of organic matter in ponds.

Mishustin (1947) showed that certain bacilli can serve as indicators of the extent of decomposition of organic matter in soil. One of these organisms is *Bacillus cereus* var. *mycoides*. This microbe appears in soil when decomposition of organic matter commences, and disappears only when decomposition is complete (Mishustin, 1947, 1954).

Bacillus species play an important role in water as well as in soil. Kuznetsov (1952) observed that the nutritive value of a pond is largely dependent upon the proportion of the sporeforming to the nonsporeforming microorganisms in it. Therefore the determinations of the species of sporeformers in pond waters carried out by ZoBell (1946), Kriss (1949), Egorova (1951), Mefedova (1955) and others are of definite significance.

The purpose of this work was to determine which *Bacillus* species are present in the Lednize fish-breeding ponds because of their importance to our fish-breeding industry.

METHODS

The water tested was obtained from three fish-breeding ponds — Nesyt, Sredny, and Mlynsky — in the vicinity of Lednize in Moravia. The total number of bacilli was obtained by plate count and the strains were isolated from the plates. Meat-peptone agar (MPA) containing beer wort — as recommended by Mishustin — was used as nutritive medium.

The water samples were heated at 80°C for 10 min before plating. The plates were incubated at 30°C for 48 hr. Colonies of different morphology were selected from the plates and identified. The following methods were used to identify the strains.

Hucker's modification of Gram's stain was used, the presence of spores was established with Peshkov's

stain and by the heat test, and motility was studied by Haines' U-tube method. Gelatin liquefaction was determined in a stab culture. Reduction of nitrates was determined in nitrate bouillon with the Griss-Ilosvaya reagent, hydrogen sulfide production — by a filter paper moistened with a saturated solution of lead acetate, indole production — with Kovacs' reagent.

Sugar fermentations were conducted in peptone water with 1% sugar and bromothymol blue, hydrolysis of starch — on starch agar which was flooded with Lugol's solution on the fourth to the seventh day of growth. Utilization of citrates was studied on Koser and Simmons' medium. Formation of acetylmethylcarbinol and the methyl red test were carried out in Voges-Proskauer's nutritive medium with Barritt's reagent and methyl red.

Production of urease was determined by the micro-method of Cowan (Clark and Cowan, 1952); tolerance to NaCl was determined in bouillon containing 5% NaCl. Hemolytic activity was studied on sheep's blood agar. The majority of the methods used are adequately described in the Manual of Microbiological Methods (1957). The strains were classified according to the system of Proom and Knight (1955) and of Bergey's Manual (1957).

The identified strains were added to the collection of microorganisms of the Department of Microbiology of the Natural Science Faculty of Brnovskii State University.

RESULTS

Sixty-two strains of the genus *Bacillus* were isolated from the Lednize fish breeding ponds and identified. The results are shown in the table. The species of bacteria are listed in the sequence encountered, beginning with the species most frequently found.

With minor exceptions, all of the bacilli which we identified showed the characteristics described in Proom and Knight (1955) and in Bergey's Manual (Breed, Murray, and Smith, 1957).

Only a few strains — *Bacillus cereus*, *B. cereus* var. *mycoides*, *Bacillus pumilus*, and *Bacillus megaterium* —

* Technical work performed by E. Benesh.

Representatives of the Genus *Bacillus*
Found in Lednize Ponds

Species	No. of strains isolated from ponds			Total no. of strains
	Nesyt	Sredny	Mlyn-sky	
<i>Bacillus cereus</i>	4	5	7	16
» <i>cereus</i> v. <i>mycoides</i>	3	8	4	15
<i>Bacillus megaterium</i>	7	3	—	10
<i>Bacillus pumilus</i>	2	4	2	8
» <i>subtilis</i>	1	3	4	8
» <i>firmus</i>	—	1	1	2
» <i>sphaericus</i>	—	—	2	2
» <i>alvei</i>	1	—	—	1

exhibited minor deviations in sugar fermentation (for example, they did not ferment galactose or fructose) and in H_2S production. These deviations, in our opinion, have no significance. They indicate differences among strains which have resulted from the influence of ecological conditions on the species mentioned. The results of our investigation confirm, to a large extent, the opinion of Mishustin (1947, 1954) that *B. cereus*, *B. cereus* var. *mycoides*, *Bacillus subtilis*, and *B. megaterium* are among the most widely distributed species of bacteria in water as well as in the soil.

In the ponds tested, no substantial difference in the species composition of the microflora was found.

B. cereus, *B. cereus* var. *mycoides*, *B. subtilis*, and *B. pumilus* were encountered in all the ponds. *B. megaterium* was not found in the Mlynsky pond. *Bacillus firmus* was not found in the Nesyt pond; *Bacillus sphaericus* was found only in the Mlynsky pond, and *Bacillus alvei* — only in the Nesyt pond. Irkova (1959) and Pelčalová (1960) obtained similar results. These authors also studied the microflora of the Lednize ponds and found the same *Bacillus* species to be prevalent in the muddy sediments of the ponds. The fact that the majority of the species cited in the table are also abundant in another type of reservoir has been established by

Egorova, for example, who found them in Lake Belovod' (1951), and by Kuznetsov (1952).

SUMMARY

Studies were made of the *Bacillus* species in the pond waters of Nesyt, Sredny, and Mlynsky near Lednize in Moravia.

Sixty-two strains which belong to the following species were isolated: *Bacillus cereus*, *Bacillus cereus* var. *mycoides*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus firmus*, *Bacillus sphaericus*, and *Bacillus alvei*.

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ROLE OF AZOTOBACTER IN BIOLOGICAL PURIFICATION OF PETROLEUM WASTE WATERS

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With the growth of industry there has been a great increase in the quantity of waste waters containing organic contaminants. Hence, considerable attention has recently been given to waste water purification, particularly biological purification.

As we know, the biochemical oxidation of substances in biological purification devices (biofilters and aeration tanks) is the result of the vital activity of microorganisms. For the growth of microorganisms the water must contain a sufficient quantity of biogenic elements (nitrogen - 20-25 mg/liter; phosphorus - 5-10 mg/liter).

Since many industrial waste waters, particularly the effluent from the electric desalting plant "ÉLOU" of petroleum refineries, contain very little nitrogen, then biogenic elements in the form of sewage liquid or mineral salts may have to be added in industrial plants. Since petroleum refineries are usually situated far from towns and settlements, it is often difficult to supply sewage water to them, whereas the addition of mineral salts is practically inconvenient.

In view of this it became necessary to conduct experiments to find out if *Azotobacter* could be used as a nitrogen fixer. The addition of mineral salts to plant waste waters could then be obviated.

The literature contains reports which indicate that *Azotobacter* can utilize various organic compounds - carbohydrates, alcohols, and organic acids - as a source of carbon (Rubenchik [1958]).

Beside the carbon source, another important factor for the development of *Azotobacter* is the presence of calcium salts and phosphates. A pH of 6.6-6.8 is most favorable for the development of *Azotobacter*.

According to Fedorov's data [1948], *Azotobacter* requires good aeration, a fixed temperature (20-25°C), neutral pH, phosphorus and a suitable source of carbon.

The literature contains no information on the use of nitrogen-fixing microorganisms in the biological purification of waste waters, apart from Izyurova's paper [1958], which indicated that the consumption of the nitrogen added in the form of salts for the biological purification of waste waters containing petroleum products could be reduced by the introduction of an *Azotobacter*

culture. This information cannot be used, however, since no experimental data on this question were given.

The present work was conducted with the waste water flowing out of the electric desalting unit of Moscow oil refinery.

The waste water used for the work contained a large amount of salts of Ca (up to 400 mg/liter) and Mg (30 mg/liter). This effluent also contained 5 to 10 mg of nitrogen per liter, and 10 mg of petroleum per liter. The biological oxygen demand (BOD) was 200 mg/liter.

In our work we used a pure *Azotobacter chroococcum* culture obtained from the Institute of Agriculture Microbiology of VASKhNIL, and dry "azotobacterin" from a bacterial fertilizer plant.

The *Azotobacter* culture was maintained in Beijerinck medium at 20-25°C.

The *Azotobacter* was added to the waste water in the form of a suspension obtained by washing out a culture from one tube. The azotobacterin was added in the form of a weighed batch.

We determined the total nitrogen, mineral nitrogen, and solid deposit in the culture, and we also counted the number of *Azotobacter* cells introduced with the inoculum. The count of the *Azotobacter* was made on Petri dishes containing solid Beijerinck medium and by the direct method in a Thoma chamber. In addition, we took separate small clots from the sludge by means of a glass rod with a drawn-out point, and distributed them on agar Beijerinck medium, on which they grew. We then noted the percentage of clots which gave a growth of *Azotobacter*.

Saprophytic bacteria were counted on meat-peptone agar. A qualitative microscopic analysis of the water and sludge was made at regular intervals.

Total nitrogen was determined by the Kjeldahl method. The other chemical analyses were performed by standard techniques.

The work was conducted along two lines: 1) laboratory experiments in microaerators with continuous aeration and 2) on experimental models of aeration tanks.

The laboratory experiments were performed in 500 ml microaerators containing an artificially prepared effluent similar to that entering the experimental aeration

Indices *	At start of expt.	Change of liquid		No change of liquid, after 10 days
		after 5 days	after 10 days	
pH	7.6	7.1	8.3	7.9
Total nitrogen, N mg/liter	9.0	24.5	35.0	31.5
Salt nitrogen, mg/liter	7.7	9.8	15.4	23.8
Suspended matter, mg/liter	26	-	-	9.5
Total BOD, mg O ₂ /liter	147	-	-	29.0
Quantity of sludge, mg	-	-	-	41.1
Nitrogen in sludge, %	-	-	-	8
Number of Azotobacter cells (direct count, per ml)	58.10 ⁴	3.10 ⁶	8.10 ¹⁰	14.10 ¹⁰
Number of colonies in Beijerinck medium (in 1 ml)	15.10 ³	1.10 ⁵	3.10 ⁶	8.10 ⁶
Number of colonies on MPA after inoculation with 1 ml	0	0	0	0

* The chemical analysis was carried out by scientific worker E. V. Kollerova.

tanks. The artificial effluent consisted of 40 ml of industrial water in 500 ml of tap water, with the addition of 2.5 mg of phosphorus (potassium phosphate); the pH was brought to 7.6-7.8. The BOD was about 200 mg/liter.

The liquid was sterilized and then the Azotobacter culture was added.

We considered it essential to find out first if a sludge floc of Azotobacter could be formed in the industrial waste water issuing from the ÉLOU. For this purpose we set up an experiment in which the control consisted of a liquid Beijerinck medium. We noted that the Azotobacter started to multiply in the artificial effluent after one day, and that compact flocs of sludge were formed after five days. The numbers of Azotobacter (i.e., individual cells and tiny flocs) increased from $675 \cdot 10^3$ to $72 \cdot 10^6$ per ml, and the zoogaea consisted of large granular cells. The quantity of total nitrogen increased from 5.04 to 12.25 mg.

The following experiments were also conducted in microaerators: A 5 ml Azotobacter suspension was added to each vessel; in one vessel the water was changed after five days, and in the other vessel it was left unchanged for ten days. Chemical and microbiological analyses were made at the start of the experiment and at fixed intervals. The results of the experiment are given in the table.

As the table reveals, after ten days the total nitrogen increased to 22 mg when there was no change of liquid. The number of Azotobacter cells increased from $58 \cdot 10^4$ to $14 \cdot 10^{10}$ per ml.

The biological oxygen demand was reduced by 88%.

After ten days with no change of liquid, 41 mg of sludge consisting of large spherical Azotobacter cells was formed. The nitrogen content of the sludge was 8%.

The results obtained indicate that an active sludge of Azotobacter can be formed in industrial waste water

issuing from the ÉLOU, and that Azotobacter can play a part in the biological purification of waste waters.

Having obtained a positive result in the microaerators, we introduced an Azotobacter culture into experimental models of the aeration tanks used for biological purification of the petroleum waste waters from the ÉLOU. As a control we used an aeration tank operating with waste water without the addition of mineral nitrogen. The effluent from the ÉLOU contained 5 to 10 mg of salt nitrogen per liter, but such a quantity of nitrogen was obviously insufficient and the purification in the control tank became unsatisfactory. After two months the outflowing water was found to contain ammonium nitrogen which had not been oxidized. The water was turbid, the sludge was puffy and settled badly. The aeration tank to which the Azotobacter culture had been added functioned normally.

The work was continued over a long period with two aeration tanks, to one of which nitrogen was added regularly, and to the other an Azotobacter culture was added (in the first period of operation of the tanks 10 ml of Azotobacter culture was added three times at intervals of 15 days and then there were no further additions for 8 months). The operation of the tanks was checked by chemical and biological analyses. The results of the chemical analyses of the water issuing from the two aeration tanks were the same. After a short time, however, and during the whole subsequent period of operation of the tanks, we noted that the sludge in the aeration tank to which the Azotobacter culture had been added settled well, was dense, compact, and much better than that in the control aeration tank. The outflowing water was clear. The total nitrogen in the sludge was 11%, as compared with 8% in the control aeration tank. The sludge accumulated rapidly. This is one of the main technical indices of the microbial assimilation of substances contained in waste waters.

From a microscopical analysis we established that the sludge consisted of large *Azotobacter* cells.

The *Azotobacter* was regularly estimated from the percentage of colony-producing clots. Usually 75-100% of the total number of clots gave a growth.

Several saprophytic bacteria grew along with the *Azotobacter* in the active sludge. These were counted on meat-peptone agar. The sludge contained considerable numbers of *Opercularia*, rotifers and several other organisms indicative of satisfactory operation of the tanks.

From a microscopical analysis of the active sludge, the main component of which was *Azotobacter*, it was easy to assess the quality of the work of the biological purification devices from the form of the sludge flocs and the morphology of the cells comprising the sludge. When the tanks were not operating properly, the shape of the *Azotobacter* cells changed.

As our investigations showed, sludge consisting of *Azotobacter* (*azotobacterial* sludge) was very resistant to drying; at temperatures up to 60°C the sludge remained viable and retained its properties.

As we know, the microbial complex of active sludge in aeration tanks is formed during the startup period of operation of the tank. It should be noted that the startup period for petroleum waste water is 1-1.5 months.

For starting the operation of the tank, in one case 50 mg of dry *azobacterin* was added for every 8 liters of waste liquid. A chemical analysis of dry *azotobacterin* showed that it contained 1.03% total nitrogen, 40.2

% ash, and 59.8% organic matter. The number of *Azotobacter* was 235 thousand colonies per ml. Microscopical examination and bacterial counts were made as the sludge formed. We found from microscopical and chemical analyses that within ten days the aeration tank was effecting oxidation and an active sludge was formed.

These results show that an *Azotobacter* culture in the form of a suspension or dry *azotobacterin* can be introduced into purification devices and that this shortens the period of ripening of the aeration tank.

SUMMARY

Investigations showed that *Azotobacter* forms an active sludge in waste water from electric desalting units of petroleum refineries and, developing in this water, assimilates molecular nitrogen.

Azotobacter, the main component of the active sludge, participates in the biological purification of waste waters.

The addition of an *Azotobacter* culture during the startup period reduces the time of ripening of the active sludge.

Such active sludge could possibly be used as an agricultural fertilizer.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. Some or all of this periodical literature may well be available in English translation. A complete list of the cover-to-cover English translations appears at the back of this issue.

A STUDY OF VITAMIN B₁₂-SYNTHESIZING CELLULOSE BACTERIA

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The principal agents responsible for the biological hydrolysis of cellulose and its oxidation to carbon dioxide gas are aerobic (myxobacteria, vibrios, sporogenous and nonsporogenous bacteria, actinomycetes, proactinomycetes, mycobacteria, etc.) and anaerobic (mesophilic and thermophilic) microorganisms (Imshenetskii, 1953).

On the other hand, it is known that soil, peat, the silt deposits of sewage, rivers, lakes, marshes and seas, manure, the gastrointestinal contents of animals, etc., contain certain amounts of vitamin B₁₂. Coates, et al. (1951, 1952), found vitamin B₁₂ in the contents of the rumen and in the feces of ruminants.

Neujahr (1955) observed that the anaerobic fermentation of sewage is accompanied by the formation of considerable amounts of vitamin B₁₂ analogs. Lochhead and Thexton (1951) report (according to Margaret Burton's unpublished data) that 70% of the bacteria isolated from cultivated soils and 84% of the bacteria isolated from uncultivated soils from northern Canada are capable of synthesizing vitamin B₁₂.

From our investigations, (unpublished), it can be seen that silt from a number of marshes and lakes in Bulgaria contains vitamin B₁₂.

Taking into consideration the presence of vitamin B₁₂ in natural materials on the one hand, and on the other the fact that a substantial portion of their bacterio-coenosis is made up of cellulose-decomposing bacteria, we consider it probable that cellulose bacteria participate, along with other microorganisms, in the biosynthesis of vitamin B₁₂ and its analogs in nature.

In order to verify this assumption, we investigated for its ability to synthesize vitamin B₁₂ the cultures of an anaerobic, Gram-negative, sporulating, cellulose-decomposing bacillus which we had isolated from soil and designated as *Bacillus Omelyanskii*. We do not accept the suggestion of Bergey, et al. (1957), of placing the taxonomic position of mesophilic, Gram-negative, anaerobic, cellulose-decomposing bacilli in the genus *Clostridium*, because they are typical plectridial forms.

We regard as correct the proposal by Clausen (1931) and by Imshenetskii (1953) that anaerobic, cellulose-decomposing bacteria be combined into a single gen-

eral group named *Bacillus Omelyanskii*, after the prominent Russian microbiologist, V. L. Omelyanskii.

MATERIALS AND METHOD

Nutrient Media

Solid nutrient media. Glucose agar containing 0.8 mg FeSO₄ and from 1.5 to 2.5% agar per liter.

Blood agar with 5% blood.

Liquid nutrient medium. Peptone water (1% peptone in phosphate buffer at pH 7.0), 0.5% of the appropriate carbohydrate, and 0.025% ascorbic acid. The aqueous solutions of the carbohydrates tested were sterilized by filtration through a Seitz filter (frame filter press), and were added to the peptone water prior to inoculation.

Ascorbic acid was used in the form of a prepared sterile 10% solution in ampules; the medium was dispensed in 3-ml amounts in sterile test tubes measuring 1 × 10 cm. No indicator was added to the medium, since it inhibits the growth of the organism.

Changes in the pH of the cultures following their incubation, as well as of control uninoculated media, were established by means of universal indicator paper ("Indical," VEB, Berlin - Chemie Adlershof).

Omelyanskii's medium, containing 0.8 mg FeSO₄ and 0.025% ascorbic acid per liter.

Liquid nutrient medium for the biosynthesis of vitamin B₁₂. Wheat bran - 50 g, Co(NO₃)₂ - 0.001 g, FeSO₄ - 0.8 mg, tap water - 1 liter. This medium was dispensed in 100 ml amounts in 200 ml Erlenmeyer flasks. Sterilization was carried out at 1.5 atm for 40 min. The final pH was 7.0-7.5. Prior to inoculation, 0.025% ascorbic acid was added to the medium.

The amount of vitamin B₁₂ in the culture was determined by the diffusion method on level agar with *E. coli* 113-3 as the test culture. For a clearer demarcation of the growth zone, 0.20% of sterile saturated aqueous solution of indicator was added to the nutrient agar according to Nachev's method (1959).

The separation and identification of the different analogs of vitamin B₁₂ was carried out by means of paper

partition chromatography with the aid of a secondary butanol - acetic acid - water solvent.

The separated analogs were developed according to the bioautographic technique on level agar containing 0.25% Chinese blue with *E. coli* 113-3 as test organism. The different analogs were identified by means of a parallel bioautogram of pure preparations of cyanocobalamin, vitamin B₁₂ III (5-hydroxybenzimidazole), pseudovitamin B₁₂, and factor B.

Catalase activity. Using a platinum loop, the agar culture was spread in a drop of 10% hydrogen peroxide solution on a slide; the appearance of gas bubbles was noted.

CULTURE CONDITIONS

Anaerobic conditions. In order to investigate colony morphology, the organism was grown anaerobically on level agar in Petri dishes according to Koch's method (1943).

To test fermentation reactions, tubes of liquid cultures, as well as one of each of the tubes containing the corresponding carbohydrate but not inoculated, were placed in a thick-walled desiccator which had pyrogallic acid and a 10% solution of potassium hydroxide on the bottom. Air was pumped out of the hermetically sealed vessel with an oil pump until gas bubbles appeared in the liquid middle.

Aerobic conditions. The ability of the organism to synthesize vitamin B₁₂ was tested under conditions of aerobic growth in liquid bran medium (free of vitamin B₁₂) at 37 C. For this purpose, the redox potential of the nutrient medium was lowered to the optimum value by the addition of 0.025% ascorbic acid.

Optimal conditions for the aerobic cultivation of *B. Omelyanskii* were produced by joint culture with a

strain of *B. mesentericus*, which has high catalase activity but which does not synthesize vitamin B₁₂. In this case, no ascorbic acid was added to the medium.

RESULTS

Colony morphology

On the third day on glucose agar plates (2-2.5% agar) under anaerobic conditions, round, slightly convex colonies grew which were milky white with a smooth, semiopaque surface and a buttery consistency. The size of the colonies varied from 1 to 6 mm in diameter (Fig. 1).

On blood agar, the organism gave diffuse growth, and hemolysis was observed around single colonies.

Cell morphology

The vegetative cells of the organism were always Gram-negative, motile, and were thin rods with one end slightly tapered; they were arranged singly, more frequently in pairs, and less frequently in chains of six to eight cells. In microscopic preparations of older cultures, vegetative cells had subpolar vacuolization of the protoplasm.

The size of the vegetative cells varied ($2-4 \times 0.4-0.8 \mu$), depending on the composition of the nutrient medium.

On stained microscopic preparations, various stages of spore formation could be observed: elongated vegetative cells with terminal thickening, slightly swollen; plectridial forms with mature terminally situated round spores; plectridial forms in which the vegetative part of the cell was in various stages of lysis; free round spores measuring 0.7μ in diameter, with a small spine on the surface of some spores (Fig. 2).

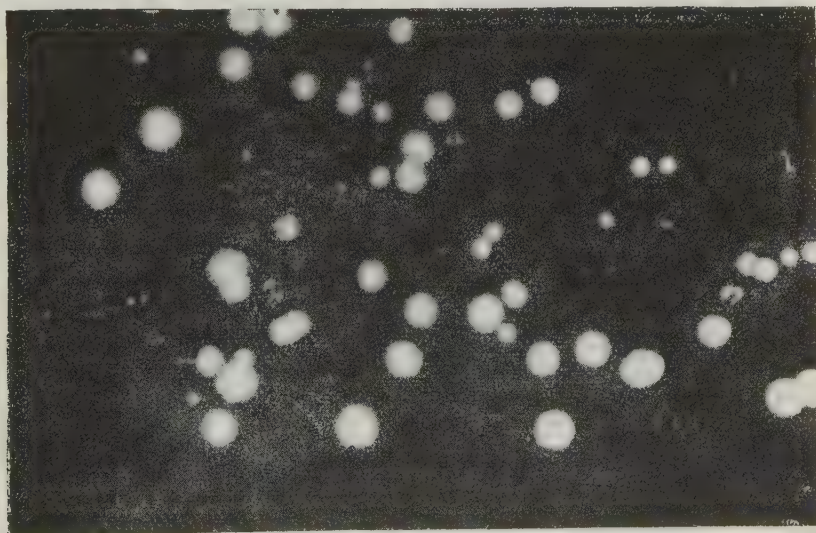


Fig. 1. Typical round colonies of *Bacillus Omelyanskii* on 2.5% glucose agar.

On shadowed electron micrographs, vegetative cells gave a relatively smaller shadow as compared with free spores or the spores of plectridial forms.

On preparations from typical round colonies, vegetative cells were observed which had a single flagellum representing a double filamentous extension of the cytoplasmic membrane. The flagellum was polar and was 1.5-2 times longer than the cell (Fig. 3).

On preparations with partially autolyzed cells, granulation and separation of the cytoplasm from the cell wall were seen. The cell wall had a double structure — an outer membrane and an inner layer (Fig. 4).

Free spores were round and gave a shadow which was two to three times larger than the diameter of the spore.

With some spores, depending on the extent of lysis of the vegetative part of the cell and on their location in the preparation, small remnants of the vegetative portion of the cell could be observed (Fig. 5). It is these remnants which appeared as small spines situated on the surface of the spore when stained microscopic preparations were examined in the light microscope.

Biochemistry

From the data presented in the table, it can be seen that the pH value of the uninoculated media containing the test carbohydrates did not change in 72 hours at 37 C. When inoculated with B. Omelyanskii, xylose, glucose, galactose, mannose, lactose, sucrose, maltose, cellobiose,

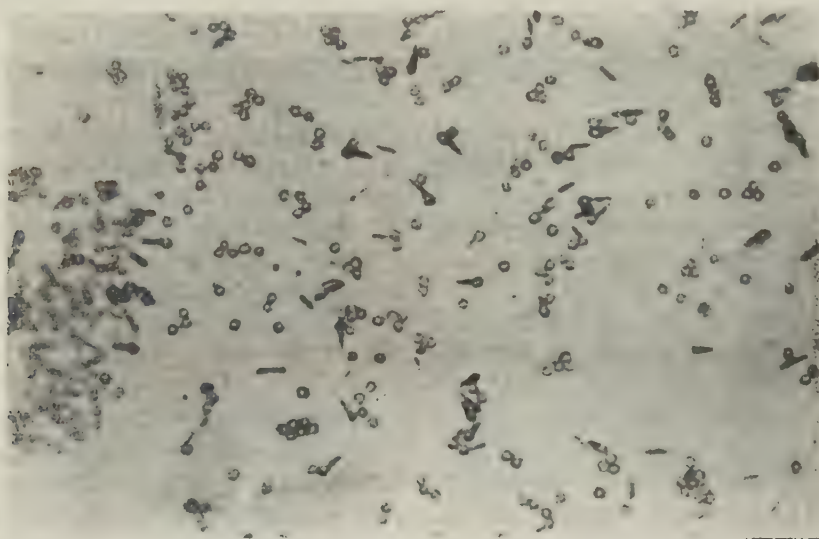


Fig. 2. Vegetative cells, plectridial forms, and free spores of Bacillus Omelyanskii. Magnification 1500 \times .

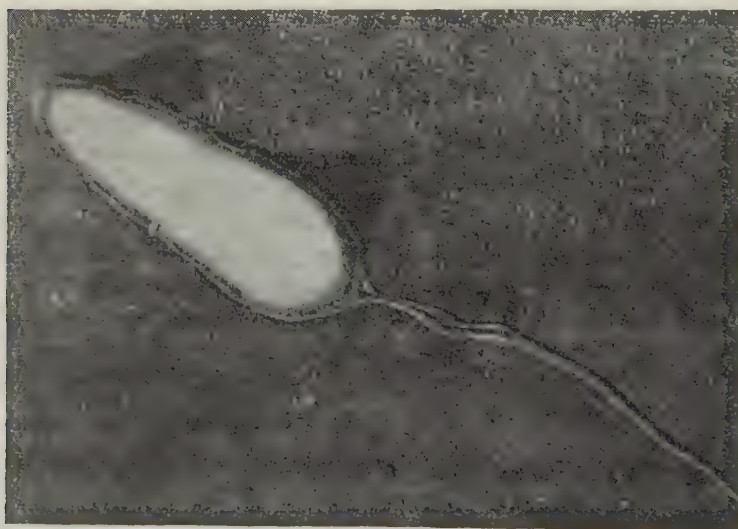


Fig. 3. Electron photograph of a vegetative cell from a typical round colony showing a single polar flagellum. Chromium shadowed; magnification 27,200 \times .

Results of Fermentation Tests (uninoculated medium pH was 7.0 at the 72nd hr)

Carbohydrate	Inoculated medium pH at 72nd hr
Arabinose	7.0
Xylose	5.9
Rhamnose	7.0
Glucose	5.9
Galactose	6.1
Mannose	6.0
Lactose	6.0
Sucrose	6.2
Maltose	6.0
Cellobiose	5.8
Trehalose	7.0
Raffinose	6.3
Soluble starch	6.2
Dextrin	6.8
Inulin	5.5
Adonitol	7.0
Mannitol	7.0
Dulcitol	7.0
Inositol	7.0
Glycerol	7.0
Sorbitol	7.0
Salicin	6.1
Melezitose	6.0
Ribose	7.0

raffinose, starch, dextrin, inulin, salicin, and melezitose were fermented with acid production; in nutrient medium containing ribose, the organism grew well without causing a change in the pH of the medium.

Pure cultures of the organism assimilated cellulose very poorly in Omelyanskii's medium.

Vegetative cells of the organism did not form catalase, did not reduce nitrates to nitrites, and did not liquefy gelatin.

Physiology

The optimal temperature for the growth of the organism is 37 C.

In monoculture, this organism grows only under anaerobic conditions. If an agent which decreases the redox potential is added to liquid nutrient media, then oxygen is not toxic for the organism, and it grows under aerobic conditions as well. The best reducing substances are ascorbic acid or catalase (blood or bacterial).

Iron and its salts in the amount of 0.8 mg/liter of liquid medium promote growth of the organism under aerobic conditions.

Biosynthesis of Vitamin B₁₂

A monoculture of B. Omelyanskii in liquid nutrient medium (bran) under aerobic conditions synthesized vitamin B₁₂ as early as 48 hr (test organism E. coli 113-3). In 72 hr, the amount of vitamin B₁₂ per liter of culture fluid was 0.566 µg.

A mixed culture of B. Omelyanskii and B. mesentericus in the same medium produced 6.540 µg of vitamin

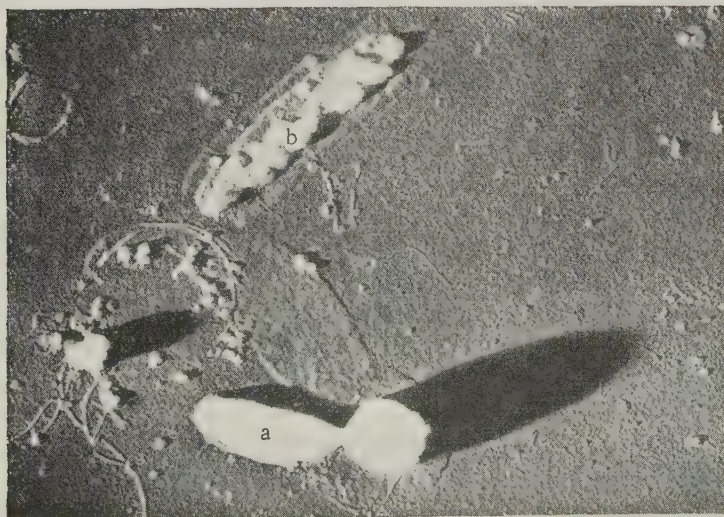


Fig. 4. Electron photograph. a) Plectridial forms with terminal arrangement of round spore; b) partially autolyzed vegetative cell with granulated cytoplasm and double-layer cell wall. Chromium shadowed; magnification 16,000 X.

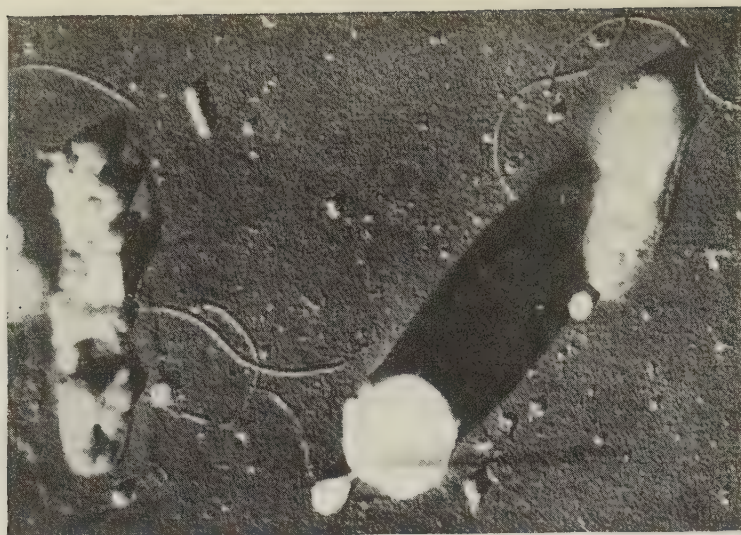


Fig. 5. Free round spore with small remnant of the vegetative portion of the cell. Chromium shadowed; magnification 24,000 X.

B₁₂ per liter of culture fluid in 72 hr, i.e., approximately 1.5 times more* in comparison with the monoculture of *B. Omelyanskii*.

The *B. mesentericus* culture alone produced no vitamin B₁₂ even as late as the 168th hr of cultivation.

By means of paper partition chromatography and the bioautographic technique, three spots corresponding to pseudovitamin B₁₂, vitamin B₁₂ III (5-hydroxybenzimidazole), and traces of cyanocobalamin (5,6-dimethylbenzimidazole), were determined both in the *B. Omelyanskii* monoculture and in its mixed culture with *B. mesentericus*.

The author expresses his gratitude to P. Kosturkova for technical assistance rendered in the performance of the tests and to I. Kalycheva for the electron photographs.

SUMMARY

A description is given of the cultural-morphological and physiological properties of the anaerobic, mesophilic cellulose bacillus *Bacillus Omelyanskii* (which can synthesize vitamin B₁₂) and its analogs in pure cultures as well as in association with *Bacillus mesentericus*.

The results of laboratory investigations give reason to assume that the culture described, when growing under natural conditions on cellulose-containing materials, not only causes the hydrolysis of the latter, but is also one of the participants in the biosynthesis of vitamin B₁₂.

In connection with this, a thorough study of cellulose bacteria, particularly of anaerobic cellulose bacilli, is of both theoretical and practical interest.

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RATE OF BIOSYNTHETIC PROCESSES DURING THE AEROBIC REPRODUCTION OF THE YEAST *Torulopsis utilis* ON MEDIA CONTAINING VARIOUS SUGARS

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The rate of accumulation of yeast biomass in a ferment is the result of the interaction of a large number of factors: the quantity of yeast per unit volume, rate of reproduction of the yeast depending on the nature of the yeast itself and physiological condition, the composition and concentration of carbon-containing, energy-yielding material (sugar, organic acids, alcohols, etc.) in the nutrient medium, the presence of biostimulants and essential mineral elements, oxygen supply, reaction of the medium (pH), and optimal temperature.

By its nature, the living yeast cell is related to open systems (Pasynskii, 1957; Ričica, 1958). The process of biosynthesis and reproduction in yeast represents a series of consecutive interrelated reactions. This entire series can be divided into external and internal reactions. External reactions, which are dependent on the entry of nutrients into the cell and the escape from it of metabolic products, are essentially osmotic in character. During the course of these reactions, the concentration gradient plays the leading role. Internal reactions, on the other hand, depend primarily on the enzymatic apparatus of the cell and on the rate constants of the individual links in the chain of coupled reactions. Hinshelwood (1946) notes that the kinetics of intracellular reactions is very conservative. This explains the difficulty and long duration of adaptive processes. This chain of internal coupled reactions is essentially what determines the rate of biomass accumulation. External reactions, on the other hand, primarily characterize the number of microorganisms involved in the process of synthesis (Kvasnikov, 1938). All of these processes are subject to the general laws of chemical kinetics.

Proceeding from these premises, the kinetics of the reproduction of yeast can be described as the kinetics of the consumption by yeast of the basic component of the substrate, such as sugar (RS), for example.

In the simplest case, when growth of the yeast occurs on a medium which contains a single carbon source, the growth is proportional to the rate of substrate utilization (Herbert, 1956; Yamada, 1956).

$$\frac{dx}{dt} = -Y \frac{ds}{dt}$$

However, along with synthetic processes, desmolytic processes accompanied by the consumption of the basic substrate also go on simultaneously with varying intensity. In addition to this, endogenous metabolism goes on constantly (Herbert), resulting in the oxidation of cellular material to CO₂.

In connection with the autoreproductive capabilities of yeast, the entire reproductive process can be compared to an autocatalytic reaction occurring at a constant rate. Consequently, the rate of biomass accumulation during the rapid course of the process can be expressed as an exponential function. However, this is possible only if the basal metabolism (endogenous metabolism) is a constant portion of the rate of substrate consumption, or if it is sufficiently small as compared with constructive metabolism.

The ratio of the two types of metabolism under the given specific experimental conditions is characterized by the efficiency coefficient α , i.e., by the increment in biomass in grams per 1 g of substrate consumed. The increment in yeast is the exponential function of the ratio of the amount of active yeast in the inoculum b to the efficiency coefficient b/α . The dimensions of this value will be:

$$\frac{b}{\alpha} = \frac{\text{g of biomass}}{\text{g of biomass/g of RS}} = \text{g RS}$$

The rate constant of biomass synthesis in yeast can be determined experimentally from the concentration curve of the residual sugar in the fermentation mixture, $S = f(t)$. In the given case, this function is expressed by the following equation:

$$S = S_i - \frac{b}{\alpha} (e^{K_s t} - 1), \quad (1)$$

where S_i is the initial concentration of sugar (RS) in g/liter; S is the concentration of sugar (RS) at end of

period of time \underline{t} , \underline{b} is the amount of active yeast in inoculum g.a.c./liter, α is the efficiency coefficient g of biomass/g of RS, and e is the base of natural logarithms; K_s is the rate constant of biomass synthesis g/g hour; \underline{t} is the time in hours.

In this equation, \underline{b} represents only those active yeasts which take part in reproduction and biomass synthesis, rather than the whole mass of inoculum yeasts. Under unfavorable conditions, activity decreases markedly, and in some cases it may become equal to zero. Then the population will only decrease due to the constant drying off of part of the cells. The value \underline{b} cannot yet be determined by direct measurement. However, it, as well as the value of the rate constant of biomass synthesis K_s , can be calculated mathematically on the basis of an analysis of curve $S = f(t)$ of the change of sugar concentration in the solution, which is plotted according to experimental data.

In order to calculate values K_s and \underline{b} , two values, S_1 and S_2 , determined by analysis during the experiment after appropriate intervals of time t_1 and t_2 from the start of the experiment, have to be substituted in equation (1).

For convenience of calculations, t_2 must be selected close to the end of the experiment, and $t_1 = t_2/2$. Then, when they are substituted in equation (1) and the system of two equations with two unknowns is solved, a first-power equation is obtained which is very easily solved. Otherwise, when $t_1 \neq t_2/2$, equations of a higher power are obtained, the solution of which presents great difficulties.

When $t_2 = 2t_1$, there is a very simple expression for the determination of K_s :

$$K_s = \frac{2.3}{t_2} \lg \frac{(S_2 - S_1)^2}{(S_1 - S_1)^2} \quad (2)$$

The amount of active yeast in the inoculum \underline{b} is determined by the equation:

$$\underline{b} = \frac{(S_1 - S_2) \alpha}{e^{K_s t_2} - 1} \quad (3)$$

After calculating values K_s and \underline{b} according to equations (2) and (3) and substituting them in equation (1), the value of function $S = f(t)$ can be computed and a curve with coordinates (S, t) can be plotted. It is obvious that if the calculated curve coincides over its entire length with the curve plotted from experimental data, it can be assumed that equation (1), which we derived, correctly expresses the essence of the observed process of biomass accumulation in yeast.

On the other hand, it is very important to establish whether or not the rate constant of biomass synthesis K_s is constant throughout the duration of the entire experiment. For this purpose, let us analyze equation (1). After conversion, equation (1) appears as:

$$(S_1 - S) \frac{\alpha}{b} + 1 = e^{K_s t}$$

Having designated $S_1 - S = \Delta S$, where ΔS is the amount of sugar (RS) consumed during time \underline{t} , we get:

$$\Delta S \frac{\alpha}{b} + 1 = e^{K_s t}$$

Converting this expression to logarithms, we get:

$$2.3 \lg \left(\Delta S \frac{\alpha}{b} + 1 \right) = K_s t \quad (4)$$

If K_s is constant, then we should obtain a straight line on semilogarithmic coordinates when the function is graphically plotted according to equation (4). If K_s does not remain constant throughout the experiment, a curved or broken line is obtained. Thus, the proposed method of mathematical analysis of experimental data enables one not only to determine the value of the rate constant of biomass synthesis, but to trace its constancy throughout the entire experiment as well.

We applied the proposed method in a study of the rate of biomass synthesis in the yeast *Torulopsis utilis* during its periodic aerobic multiplication on synthetic nutrient media containing various sugars and their mixtures.

EXPERIMENTAL*

As is well known, it is essential to ensure the greatest possible reproducibility of the experimental results in conducting kinetic investigations. Therefore, special attention must be accorded to the methodology of the investigation.

In order to ensure constant experimental conditions when carrying out growth experiments with yeast, we set up special laboratory equipment which enabled us to conduct both intermittent and continuous reproduction of yeast. The equipment consists of four fermenters, each measuring 1.5 liters in volume, placed in one common water bath, and of special measuring devices for delivering nutrient medium to the fermenters during continuous reproduction.

The arrangement of the fermenter is shown in Fig. 1, where its over-all appearance is represented in cross section. In the water bath in which the fermenters assembled for the experiment are set up, the required temperature is maintained with an accuracy of 0.2° by means of an electronic thermoregulator. Compressed air for the aeration of the fermenters is delivered from a compressor. The amount of air delivered is regulated according to the readings on rheometers installed separately on each of the fermenters.

* The following participated in performing the experiments: N. S. Nikitina, É. A. Bobereko, S. V. Belyaev, V. I. Balanin, V. I. Ryabovich, A. V. Rezhukhina, S. V. Khudchenko and L. V. Timofeeva.

It was established by means of preliminary experiments that the number of rotations of the stirrer and the amount of air bubbled through had an effect on the yeast's yield and reproduction rate which was significant only to 600 rpm of the stirrer and to 1 liter/min of air intake. Therefore, in all further experiments, the number of rotations of the stirrer was kept constant at 900 rpm, while the air supply was maintained at 2 liters/min. The pH of the medium was determined at the start and at the end of the experiment by means of a potentiometer with a glass electrode; during the experiment the pH was measured with indicator paper.

In order to eliminate errors due to evaporation of moisture during the bubbling of air, the fermenters were disconnected from the motors and weighed on technological scales accurate to 0.1 g prior to sampling. The decrease in weight of the fermenter due to evaporation was compensated by the addition of sterile distilled water.

The samples collected were immediately subjected to chemical analysis and microscopic examination. The following were determined in the samples:

1. Total RS content by the ebullistatic method of V. K. Nizovkin and I. Z. Emel'yanova with end titration with a standard glucose solution.
2. Amount of absolutely dry biomass by means of drying the residue on a filter to constant weight at 100 C.
3. Yeast cells (budding, dead, and total number) were counted in a Thoma chamber.
4. The total nitrogen content of the yeast was determined by the Kjeldahl method.

5. The phosphorus content of the yeast was determined according to Briggs with the aid of an electrocolorimeter.

6. The amount of yeast was determined by the weight method (moist and absolutely dry yeast). The entire fermentation mixture was centrifuged, and the sediment was washed with water and suctioned through a funnel. In the calculation of yeast yield, the amounts of RS and yeast removed with the samples during the experiment were taken into account.

7. During the experiment, the pH in the fermenters was kept constant by the addition of 10% NaOH solution.

The Dependence of the Rate Constant of Biomass Synthesis, K_s , on the Substrate Concentration and Amount of Inoculum

In order to determine the dependence of the rate constant of biomass synthesis, K_s , on the substrate concentration and amount of inoculum, experiments were set up on synthetic media with glucose, containing 2 and 4% glucose and 2 and 4% (by volume) of yeast autolyzate.

The yeast *T. utilis* P-1 was used as inoculum. In experiments Nos. 24, 26, and 35, inoculations were made by washing off two-day-old cultures from two tubes of wort agar slants. In experiments Nos. 28 and 29, fresh yeast obtained in experiments Nos. 24 and 26 was used for inoculum. The yeast was centrifuged, washed, filtered through a paper filter, and dried off by squeezing between two sheets of filter paper.

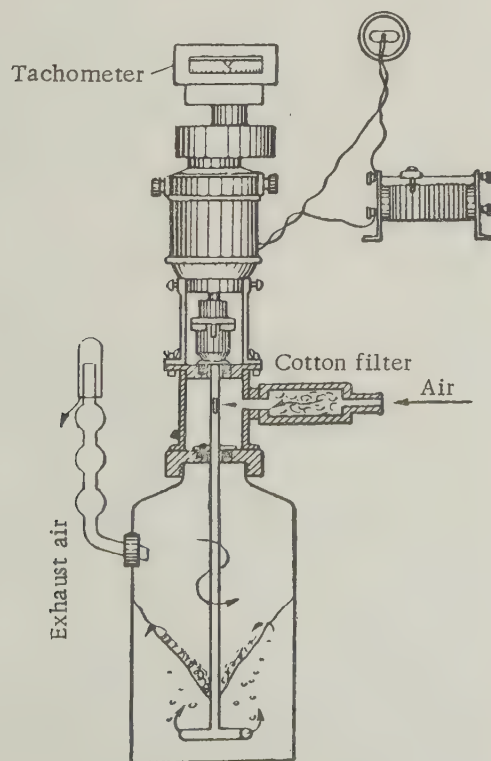


Fig. 1. Diagram of laboratory fermenter (cross section).

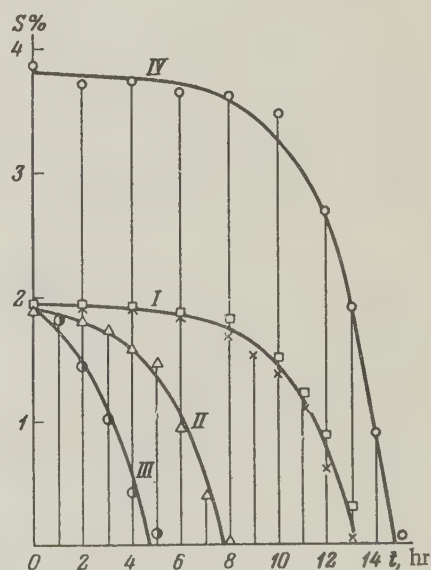


Fig. 2. Glucose consumption at various concentrations of glucose and with different amounts of inoculum. I) Experiments 24-25; II) experiment 28; III) experiment 29; IV) experiment 35; curves - calculated data; points - experimental data.

TABLE 1. Values of K_s and \underline{b} , Calculated on the Basis of Experimental Data during the Multiplication of *T. utilis* on Synthetic Medium Containing Glucose and Yeast Autolyzate

Expt. No.	Initial RS concentration in %, S_i	Amt. of inoculum, g of absolutely dry wt. per 100 ml, g	Efficiency coefficient g/g, α	Amt. of active yeast in inoculum, g absolutely dry wt. per 100 ml, \underline{b}	Rate constant of bio-mass synthesis, K_s	Duration of experiment, hr, \underline{t}	Activity of yeast in %, $\frac{100 \cdot b}{g}$
24-26	1.94	Streak washing	0.542	0.0043	0.416	13.2	—
28	1.92	0.045	0.492	0.0369	0.420	7.7	82.0
29	1.92	0.221	0.501	0.1535	0.420	4.6	69.5
35	3.86	Streak washing	0.499	0.0040	0.420	14.8	—

TABLE 2. The Values of K_s and \underline{b} Calculated from Experimental Data during the Growth of *T. utilis* on Synthetic Medium without Yeast Autolyzate

Expt. No.	S_i	g	α	\underline{b}	K_s	\underline{t}	$\frac{100 \cdot b}{g}$
44	1.94	5.1 million cells per 1 ml	0.459	0.0105	0.369	12.4	—
Average of 59-61	1.98	0.048	0.421	0.0350	0.369	9.2	73.0

In experiment No. 28, 0.5 g of pressed yeast from experiment No. 24, which had a moisture content of 77.5%, was used for 250 ml of medium. In experiment No. 29, 2.5 g of pressed yeast from experiment No. 26, having a moisture content of 77.9%, was used per 250 ml of media. The yeast was grown at 32 C. The amount of air bubbled through was 2 liters/min, the number of rotations of the stirrer was 900 rpm, and the pH was 3.8-4.2.

The results of the determination of RS content in the fermenters is represented by points (Fig. 2). After the end of the experiment, the yield of yeast and the efficiency coefficient α were determined in all fermenters. Then the values K_s and \underline{b} , which are given in Table 1, were calculated by means of equations (2) and (3).

Next, curves of RS utilization during the experiment were computed and plotted on the basis of the values of K_s and \underline{b} obtained and by equation (1). These curves are plotted as solid lines in Fig. 2. As can be seen, they coincide fairly closely with the experimental points.

The data in Table 1 show that doubling the substrate concentration (from 1.92 to 3.86%) and increasing the amount of inoculum forty-fold (from 0.004 to 0.1535 g) had no effect on the value of the rate constant of biomass synthesis, which remained constant at 0.420.

The Effect of Biostimulants on the Value of the Constant K_s

In order to determine the effect of biofactors on the value of the rate constant of biomass synthesis, experiments were set up in which the yeast *T. utilis* P-1 was grown on synthetic medium with 2% glucose, but without

the addition of yeast autolyzate. In other respects, the design of the experiments and their processing was the same as in the preceding series.

The results of these experiments are given in Table 2.

By comparing the data in Tables 1 and 2, it can be seen that, in the absence of biostimulants (yeast autolyzate) from the nutrient medium, the rate constant of biomass synthesis decreased from 0.420 to 0.369, which is quite according to pattern. The rate constant of biomass synthesis remained constant in this series of experiments as well.

The Effect of the Type of Sugar on the Value of Constant K_s

There are many data in the literature (Fenc1 and Burger, 1958; Todt and Damaschke, 1957) confirming the order and different rates of utilization of various sugars by yeasts. However, we found no data in the literature on whether the rate of utilization of individual sugars from their mixtures is an additive property.

The determination of growth rates of yeast on mixed sugars consumed at different rates (such as a mixture of hexoses and pentoses, for example) is of particular interest for industry.

In order to clarify this question, we set up experiments on the reproduction of the yeast *T. utilis* P-1, first on synthetic medium with xylose, and then on synthetic medium containing a mixture of the sugars glucose and xylose. The concentration of sugar was about 2% in all experiments. The sugar mixture consisted of equal parts

TABLE 3. K_s and b Values during the Multiplication of *T. utilis* on Synthetic Medium Containing a Mixture of Glucose and Xylose

Expt. No.	Designation of curve in Fig. 3	Composition of medium	S_1	b	α	K_s
61	I, I_a	Glucose	2.01	0.0325	0.415	0.359
34	II, II	Xylose	1.94	0.0350	0.485	0.175
50	III, III_a	Glucose +	2.12	0.0333	0.456	0.348
		+ Xylose	(0.96)	0.3970	0.456	0.160

of glucose and xylose. All experimental conditions remained as before and were kept constant.

The total RS content of the fermenters was determined every two hours in the experiments. The experimental results were treated as before. In Fig. 3, the experimental data on the basis of which K_s and b were calculated are plotted as points, and curves $S = f(t)$ are constructed. Under the same figures, using the indices I_a , II_a and III_a , curves of the function $\lg(\Delta S a/b + 1) = f(t)$ are given for these experiments. The calculated data pertaining to this series of experiments are given in Table 3.

In Fig. 3, lines I_a and II_a proved to be straight. This is evidence of the fact that the constant of the rate of biomass synthesis remained the same for the entire duration of the experiment. In experiment 50, line III_a was found to be broken. The break in this line at point C suggests that the rate constant, K_s , changed rapidly in value at this point. If point C is projected on curve III, it will be found that the change in the rate constant of biomass synthesis coincides quite closely with the moment at which all of the glucose had been utilized and only xylose remained in the fluid.

Using equation (2), we calculated the K_s values for both portions of curve III: from the start to point C, and from point C to the end of the experiment, i.e., for the periods when only glucose and only xylose were being

consumed. The constants calculated (Table 3) coincided fairly closely with the limiting constants for the rate of biomass synthesis on solutions of the pure sugars.

This result was regularly repeated in all of the eight analogous experiments which we set up with various amounts of yeast inoculum b .

In the next series of experiments on the reproduction of yeast on a mixture of glucose and xylose, the inoculum used was first grown on nutrient medium containing xylose for the purpose of adaptation to this sugar. In this case, not one, but two, breaks – at points A and B (Fig. 4) – were found on curve $f(t) = \lg(\Delta S a/b + 1)$. This is evidence of the fact that yeast which is adapted to xylose begins to consume it before all of the glucose in the solution is consumed. The last portions of glucose are consumed by the yeast together with xylose; moreover, the rate constant of biomass synthesis was found to be lower than for glucose, but higher than for xylose. When all of the glucose was consumed, the further utilization of xylose went on at a rate equal to its utilization in pure solutions.

SUMMARY

1. A criterion has been proposed for evaluating the rate of biomass accumulation during the periodic aerobic reproduction of the yeast *T. utilis*, i.e., the rate constant of biomass synthesis, K_s , which is constant for this species of yeast under certain conditions of cultivation.

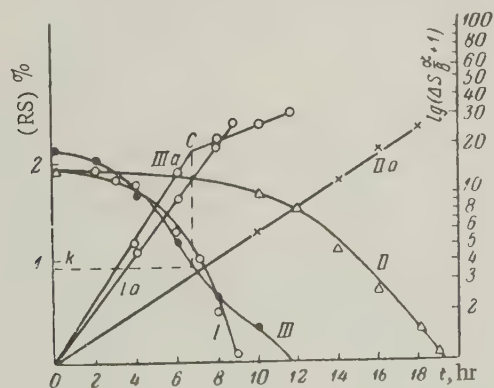


Fig. 3. Cultivation of yeast on various sugars. I) Experiment in which yeast multiplied on synthetic medium with glucose; II) on synthetic medium with xylose; III) on synthetic medium with a mixture of the sugars glucose and xylose.

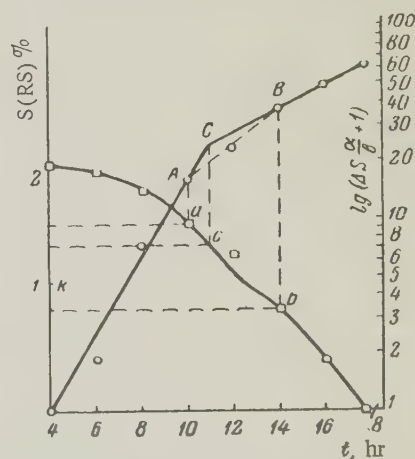


Fig. 4. Cultivation of yeast on a mixture of the sugars glucose and xylose. Small inoculum consisting of adapted yeast (experiment 49).

2. The constant K_s is determined on the basis of experimental data; from the rate of decrease of an essential component of the substrate, such as sugar (RS), for example; from the efficiency coefficient, α ; and from the quantity of active yeast in the inoculum b .

3. The value of constant K_s does not depend on the concentration of substrate and on the amount of yeast, and remains constant throughout the entire experiment.

4. The presence of biostimulants (yeast autolyzate) in the medium increases the rate constant of biomass synthesis K_s .

5. The value of constant K_s depends on the nature of the sugar. When yeast is multiplying on medium which contains glucose, K_s is twice as high as on xylose-containing medium.

6. During the multiplication of yeast on a mixture of sugars (glucose + xylose), the first sugar to be consumed is that which is utilized with the highest rate constant in pure form (glucose).

7. Each sugar in the mixture is utilized at a rate similar or equal to that of its utilization in pure form.

8. If the yeast is not adapted to xylose, then during its multiplication on a mixture of sugars, the transition

from glucose to xylose utilization is accompanied by a sharp change in K_s . During the multiplication of yeast adapted to xylose, this transition occurs with a constantly changing rate constant of biomass synthesis.

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FERMENTATION OF MIXTURES OF PENTOSE-HEXOSE HYDROLYZATES FROM PLANT WASTES AND MOLASSES BY BUTANOL-FORMING BACTERIA

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The production of acetone and butyl alcohol is accomplished in the USSR by means of the fermentation of wheat, corn, or rye flour by Clostridium acetobutylicum. Disadvantages of this process are the large expenditure of valuable nutritive raw material and the comparatively low yield of the basic fermentation product, butyl alcohol (18-19% of the fermented sugar).

A surplus of protein, necessary for normal acetone-butanol fermentation, is present in flour mashes; thus the fermentation can occur when part of the flour is replaced by other carbohydrates. This was demonstrated by Shaposhnikov (1948), who substituted part of the sugar in the flour with molasses or hydrolyzates.

The fermentation proceeded normally when part of the corn flour was replaced with molasses until approximately 55% of the total sugar of the medium was present as molasses (Logotkin, 1958).

Investigation of the possibility of replacing food materials with pentose hydrolyzates of corn cobs indicated that up to 50% of the flour can be replaced by hydrolyzate without decreasing the yield of solvent and the rate of fermentation (Nakhmanovich, 1957; Nakhmanovich and Shcheblykina, 1960).

Different results can be obtained if the fermentation organism is the butanol-forming bacterium isolated and studied by the Institute of Microbiology of the Ukr. SSR Academy of Sciences (Lipshits, 1958; Lipshits and Kolchinskaya, 1959).

Butanol-forming bacteria, according to a number of criteria (morphological, cultural, biochemical and serological), are related to the butyric acid bacterium Clostridium butyricum Prazmowsky, but differ from the latter in their ability to form primarily butyl alcohol (up to 80% of the total solvents on a potato medium—Lipshits, 1958). No isopropyl alcohol, such as Clostridium butylicum Beijerinck-Donker forms, was found among the products of fermentation.

The investigated bacteria are characterized by high amylolytic activity and the absence of proteolytic prop-

erties; they do not liquefy gelatin, or cleave potato, rye, or corn protein; this distinguishes them from the true acetone-butanol-forming bacteria. On grain media their energy for multiplication is decreased, solvents are formed in small quantities or not at all (Bergey, 1957; Prevot, 1957).

To determine what sources of nitrogen nutrition were required for these bacteria and what effect these substances had at different stages of their life cycle, their nitrogen metabolism was studied during growth of the bacteria on natural media (potato and rye), as well as on defined media. It was found that in order to form butyl alcohol, six amino acids were of great significance— aspartic and glutamic acids, lysine, valine, alanine, cysteine—each of which stimulated the formation of butanol.

A feature of our butanol-forming bacterium was the possibility of using non-protein media not consisting of foodstuffs for fermentation—hydrolyzates of various agricultural waste products. Good results were obtained from the fermentation of hydrolyzates combined with molasses.

Experiments showed that in media of molasses without hydrolyzates, the bacteria did not ferment as well as those with corn cob hydrolyzates or sunflower hulls. Hydrolyzates, consequently, served not only as a cheap source of sugars, but also of other necessary nutritive substances and stimulants of fermentation. However, the medium was still not sufficiently rich in nitrogen and phosphorus. The necessary salt supplements and their optimum dosage were established experimentally; ammonium sulfate (0.1%) and 0.04% superphosphate were added to the media.

EXPERIMENTAL

Hydrolyzates of corn cobs and sunflower hulls obtained by our hydrolysis processes (Nakhmanovich, 1957; Nakhmanovich and Shcheblykina, 1960) were used in the experiments, as well as pentose-hexose hydrolyzates obtained by Rizhskii's method of hydrolysis (Kal'nina and co-workers, 1957 and 1959). The pentose hydroly-

TABLE 1. Fermentation of a Medium Composed of Pentose-Hexose Corn Cob Hydrolyzate Combined with Molasses at Different Sugar Concentrations

Sugar concentration, %			Solvents, g/liter				Mash acidity, ml 0.1 N NaOH per 10 ml	Solvent yield, % sugar			
initial	residual	fermented	acetone	butanol	ethanol	total		acetone	butanol	ethanol	total
2.27	0.16	2.11	1.46	5.91	0.79	8.16	0.8	6.43	26.00	3.48	35.91
2.83	0.16	2.67	2.07	7.26	0.28	9.61	0.7	7.32	25.65	0.99	33.96
3.40	0.22	3.18	2.85	8.55	0.89	12.29	0.7	8.38	25.14	2.61	36.13
3.90	0.28	3.62	3.36	9.62	1.06	14.04	0.6	8.61	24.66	2.71	35.98
4.54	0.51	4.03	3.19	10.66	0.75	14.60	0.9	7.02	23.48	1.64	32.15
5.02	1.08	4.01	2.80	10.48	0.98	14.26	1.1	5.50	20.59	1.92	28.01
5.65	1.23	4.42	2.97	11.18	0.98	15.13	1.1	5.26	19.78	1.73	26.77

yzates contained — as percentages of the total sugar — 70-75% xylose, 15-25% arabinose, 2.5-5.0% glucose and 2.5-5.0% galactose. The hexose hydrolyzates were composed of 90% glucose, 6-8% xylose and 2.5-3.0% mannose.

Determination of amino acids by radial paper chromatography indicated the presence in pentose hydrolyzates of aspartic and glutamic acids, lysine, α - and β -alanine, histidine, glycine, proline, serine, tyrosine, cysteine, cystine, arginine, and also glutamine and asparagine.

The molasses used contained 44% sucrose, 1.7% total nitrogen and 6.0% ash. The following nitrogen-containing substances were present: asparagine, glutamine, aspartic and glutamic acids, lysine, valine, α - and β -alanine, and other amino acids.

The hydrolyzates were neutralized with lime to a pH of 6.7 to 6.9, filtered and mixed with molasses in amounts required to bring the total concentration of sugar in the medium to 3-4%. After sterilization (112C

for 30 min) the medium was inoculated with a second generation 12-18 hr butyl-forming culture (strain Nos. 65 and 94) to the amount of 2-3% of the volume of the medium.

The fermentation was conducted in different experiments at temperatures of 32C to 37C. The rate of fermentation was determined by gas evolution. Optimal Sugar Concentration in the Media

The optimal concentration of sugar in the original mash was investigated on media containing 30% pentose-hexose hydrolyzate of corn stalks and 70% molasses (percentages based on sugar). Concentrations ranging from 2.3-5.6% sugar were tested at intervals of 0.5%. The fermentation was carried out at 33C, using a 2.5% inoculum (Table 1).

The optimal concentration of sugar in the mash appears to be 3.9%. The total yield of solvents in this case consisted of 38% of the sugar present in the medium, but the yield of butanol was 26%. On further increase in the sugar concentration, the yield of each of the solvents decreased relatively, and the residual sugar was increased. The limiting concentration of butanol in the mash consisted of 11.5 g/liter. The fermentation was continued for 66-72 hr.

Kinetics of Fermentation

Results of an investigation of the fermentation kinetics on media consisting of 70% molasses and 30% pentose hydrolyzate of sunflower hulls (Fig. 1) or corn cobs (Fig. 2) give the overall characteristics of the process of fermentation as well as a comparison of the different hydrolyzates. The initial concentration of sugar was 3.0%. The medium was inoculated with a 12-hr butanol-forming culture as 2.5% of the volume of the medium. The fermentation was carried out at 33C. Every 4 hr a sample of the mash was removed and analyzed for sugar (after hydrolysis), titratable acidity, pH, solvent content and bacterial count.

* Investigations being carried out at the present time have shown that the use of a specially prepared medium of hydrolyzates and molasses can make it possible to raise the concentration of fermentable sugar to 5.5%. The results of this work will be presented in a separate communication.

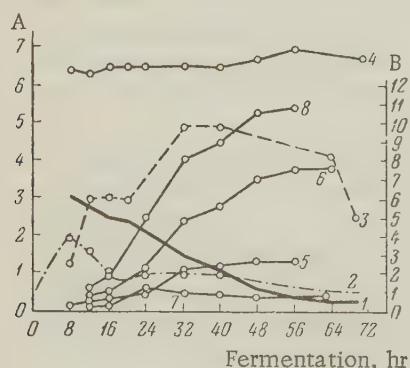


Fig. 1. Kinetics of fermentation of pentose hydrolyzate of sunflower hulls mixed with molasses (ratio of sugar 30:70).

From ordinate A: 1) total sugar (% reducing sugar after inversion); 2) acidity (ml 0.1 N in 10 ml); 4) pH. From ordinate B: 3) number of bacteria ($n \cdot 10^8$ per 1 ml); 5) acetone (g/liter); 6) butyl alcohol (g/liter); 7) ethyl alcohol (g/liter); 8) total solvents (g/liter).

TABLE 2. Amino Acid Composition of the Medium Before and After Fermentation

Amino acid	Pentose hydrolyzate of corn cobs, 30%, and molasses, 70%			Pentose hydrolyzate of sunflower hulls, 30%, and molasses, 70%		
	before fermentation	after fermentation		before fermentation	after fermentation	
		strain 65	strain 94		strain 65	strain 94
Glycine	+	+	+	+	—	—
α -Alanine	+	trace*	trace	+	—	trace
β -Alanine	+	—	—	+	—	—
Serine	+	+	—	+	—	+
Cysteine	+	—	—	—	—	trace
Cystine	+	—	trace	+	—	—
Valine	+	—	—	+	—	—
Asparagine	+	—	—	+	—	—
Aspartic acid	+	—	—	+	—	—
Glutamine	+	—	trace	+	—	—
Glutamic acid	+	trace	—	+	trace	trace
Arginine	+	—	—	—	—	—
Lysine	+	—	—	+	—	—
Hydroxyproline	—	+	—	+	—	—
Tyrosine	+	trace	+	+	+	—
Tryptophan	—	+	—	—	trace	+
Histidine	+	—	—	+	—	—
Leucine	+	—	—	+	—	—

* Trace — corresponds to a small diameter and light stain intensity of the spot on the paper chromatogram. — Not found.

TABLE 3. Fermentation of the Medium with Different Ratios of Hydrolyzate and Molasses

Hydrolyzate	Hydrolyzate/molasses ratio (based on sugar)	Fermentation temperature	Initial % total sugar in medium	Residual sugar, %	Mash acidity, ml 0.1 N NaOH/10 ml	Solvents, g/liter				Fermentation, hr
						acetone	butanol	ethanol	total	
Pentose-hexose corn cobs	15 : 85	32	3.5	0.24	0.7	3.26	9.68	0.64	13.58	83
	15 : 85	37	3.5	1.45	2.0	1.53	5.62	0.49	7.64	
	25 : 75	32	3.5	0.26	0.7	3.40	9.39	0.80	13.59	83
	25 : 75	37	3.5	1.30	1.8	1.78	5.86	0.68	8.32	
	40 : 60	32	3.5	0.34	0.5	3.60	8.96	1.25	13.81	65
	40 : 60	37	3.5	0.28	0.6	3.85	8.36	1.18	13.39	65
	40 : 60	33	4.0	0.34	0.5	4.14	8.87	2.65	15.66	66
	50 : 50	33	4.0	0.40	0.4	4.29	9.03	1.59	14.91	60
	60 : 40	33	4.0	0.45	0.4	4.24	8.74	1.75	14.73	66
	70 : 30	33	4.0	0.55	0.4	4.34	7.91	2.91	15.16	66
Pentose-hexose sunflower hulls	80 : 20	33	4.0	0.56	0.6	4.44	7.90	1.57	13.91	84
	100	33	4.0	1.73	0.7	2.96	5.22	1.71	9.89	
	20 : 80	37	3.5	0.54	0.7	2.87	5.80	2.15	10.82	48
	40 : 60	37	3.5	0.36	0.5	3.40	7.28	2.22	12.90	54
	100	37	3.5	0.54	0.6	3.50	6.25	1.69	11.44	48

The double-phase character of the fermentation as described by Shaposhnikov (1948) for acetone-butanol fermentation was clearly evident. In both experiments, the break in the acidity curve occurred 8 hr after the inoculum was introduced. Formation of the solvents began with the second phase of fermentation. The specific

content of butanol in the fermentation process increased, attaining 70% of the total solvents. The total yield of solvents was 37-38%, and butanol about 26%, from the original sugar.

This fermentation, as distinguished from the acetone-butanol fermentation, occurred at a higher pH, equal to

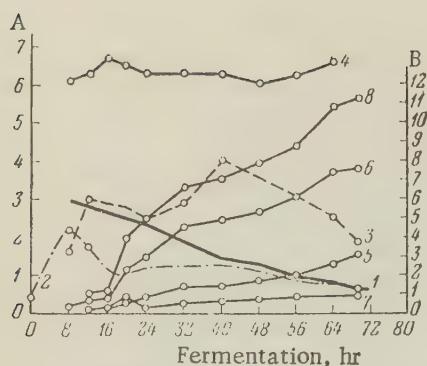


Fig. 2. Kinetics of fermentation of pentose corn cob hydrolyzate mixed with molasses (ratio based on sugar 30:70). Designations the same as in Fig. 1.

6.5. At the end of fermentation the pH had increased to 6.8, and the acidity of the mash decreased to 0.5-0.8 ml 0.1 N NaOH per 10 ml. The number of bacterial cells at the break in the acidity curve was $0.5-0.6 \times 10^9$ per ml, and after 30 hr fermentation had increased to $0.8-1.0 \times 10^9$ and remained at this level until the end of the fermentation, after which it decreased sharply.

Changes in the amino acid composition of the medium as a result of fermentation are presented in Table 2. The requirement for these same amino acids by butanol-forming bacteria was found by investigating the culture liquid on potato and grain media (Kolchinskaya, 1959; Lipshits and Kolchinskaya, 1959).

Fermentation of the Medium with Different Ratios of Hydrolyzate and Molasses

The ratio of hydrolyzate and molasses can vary within wide limits (Table 3). The medium most favorable for fermentation was that containing 40-60% hydrolyzate. With less (15-25%) and more (80%) hydrolyzate the fermentation was retarded or took place with a decrease in the yield of solvents. Media containing 15-25% corn cob hydrolyzate was not completed at 37°C; the fermentation stopped when the residual sugar was 1.45 and 1.30%. At 32°C the fermentation of these media took place normally, but lasted for 83 hr. When the hydrolyzate was increased to 40% the fermentation at both temperatures was complete at 65 hr. Hydrolyzates without molasses in the majority of cases were not completely fermented. There were experiments, however, in which the hydrolyzates underwent normal fermentation without molasses, as is shown for hydrolyzates of sunflower hulls (Table 3).

SUMMARY

1. Butanol-forming bacteria without proteolytic activity ferment media that do not contain foodstuffs and

consist of a mixture of corn cob hydrolyzates or sunflower hulls and molasses containing the amino acids necessary for fermentation. The fermentation occurred normally, from 30 to 70% of the sugar of the combined medium being furnished by the hydrolyzate.

2. The total yield of solvents was 36-38%, and the yield of butyl alcohol was 24-26% of the sugar in the medium; the concentration of the sugar in the fermentation medium was 3.5-4.0%. The fermentation continued for 60-70 hr.

3. An investigation of the kinetics of fermentation showed that the fermentation is double-phased, takes place at a high pH (6.5-6.8) and finishes at a lower acidity (0.4-1.0 ml NaOH 0.1 N per 10 ml). The content of butanol in the process of fermentation increased from 40-45 to 70% of the total solvents. The number of bacterial cells attained a maximum of about 1 billion per ml after 30 hr of fermentation.

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A HEAT-RESISTANT LACTIC ACID BACILLUS – THE CAUSE OF EXCESSIVE ACIDITY OF MILK PRODUCTS

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One of the present research problems arising in the industrial manufacture of sour milk products is that of the causes of the excessive increase of acidity in such products as cottage cheese, choice sour cream, etc. The development of this defect leads to a great deterioration in the quality of the product and causes considerable loss to the dairy industry.

Investigating the reasons for this effect, we found that excessively acid sour milk products obtained in industrial conditions contained foreign bacilli in addition to the microflora added with the starters.

Lactic acid streptococci, which are the only component of the starters used in the preparation of cottage cheese and sour cream, are characterized by a relatively low limit of acid production in the milk (120-130°T). This provided a basis for the establishment of acidity standards for these products (not more than 225°T for cottage cheese, and not more than 90°T for choice sour cream). In industrial conditions the acidity of cottage cheese often reaches 230-280°, while that of choice sour cream often reaches 95-100°. These values considerably exceed the limits permitted by the standard. In view of the regular occurrence of bacilliform microflora in such products we could regard it as the probable cause of this defect.

It was natural to assume that this microflora belongs to the group of lactic acid bacteria, since the main product accumulating as a result of its activity is lactic acid, a fact which was confirmed by a qualitative and quantitative analysis.

In the literature we have found no information on the occurrence and role of lactic acid bacilli in the manufacture of sour milk products. According to the data of Korolev [1940] and Voitkevich [1948] thermophilic lactic acid bacilli occur in small numbers in raw and pasteurized milk.

Up till now the purpose of examinations of the residual microflora of pasteurized milk has been to ascertain the destruction of pathogenic microorganisms in the milk and to discover groups of microbes which might affect the subsequent preservation of the pasteurized milk at relatively low temperatures (minimum 6-8°C, maximum 20-25°C). The main role in improving the

quality of milk during preservation, according to published data, can be ascribed to thermophilic lactic acid streptococci which are found in large numbers when pasteurized milk is plated out on solid media (Voitkevich [1948]; Bogdanov [1957]; Abd-El, Malek and Gibson [1948]; Alexander and Higginbottom [1953]).

The thermophilic lactic acid bacilli, which are poorly represented in pasteurized milk and which have a high temperature optimum of development and do not grow on the usually employed media (Voitkevich [1948]), could not interest investigators as industrially harmful.

However, in cases where pasteurized milk is used for the preparation of sour milk products and, hence, is maintained at elevated temperatures (30-50°C) after the addition of the starter, this group may have a pronounced effect on the nature of the microbiological processes occurring in these products, as is the case in cheese-making (Korolev [1940]).

The present work had the following aims: 1) to devise a method of isolating the lactic acid bacilli responsible for the excessive rise of acidity in sour milk products; 2) to study the properties of the isolated lactic acid bacilli and to identify them; 3) to study the occurrence of the lactic acid bacilli in the raw material, products and equipment and to discover the main conditions leading to their increase in industry.

METHODS

As isolation media we used sterile fat-free milk, meat-peptone agar, and agar containing hydrolyzed milk (Skorodumova [1949]; Bogdanov [1957]). The cultures were incubated at two temperatures – 37°C (temperature adopted in the State standard for determination of total numbers of bacteria in milk) and 45-50°C.

The samples for investigation, collected in a sterile vessel, were inoculated onto solid media and subsequently incubated at the corresponding temperatures. The grown colonies were then examined by microscope.

At the same time sterile milk was inoculated with the same samples and was then incubated for 16-18 hr. One to three further passages through milk were made. When examination by microscope showed that the milk was considerably enriched in bacilliform microflora, the milk was plated out on agar and incubated at the stipu-

lated temperatures. The grown colonies were examined under the microscope and when colonies typical of lactic acid bacilli were found they were isolated in sterile milk.

In the study of the contamination of equipment, it was wiped with a sterile wad of cotton, which was then put into sterile milk, incubated at the temperatures indicated above for 16-18 hr, and then examined under the microscope. Further isolation was carried out as described above.

RESULTS

In the course of the isolation work we found that the most important factors for the successful isolation of lactic acid bacilli from milk products were the choice of medium and incubation temperature. The best medium for isolation was agar containing hydrolyzed milk (pH=7.0), and the most favorable temperature for cultivation was 45-50°C.

	Optimum	Minimum	Maximum
Growth in milk	45-50	20	60-65
Acid production	45-50	20	60
Growth on agar	45	30	55

The lactic acid bacilli produced no growth on meat-peptone agar. A temperature of 37°C was also unsuitable for the demonstration of bacteria of this group either in milk or on solid media. This may partially explain why the bacilliform lactic acid microflora is overlooked in milk inspection conducted by GOST methods. Moreover, in view of the small numbers of these organisms in milk, they can only be detected in samples of milk, raw or pasteurized, after a preliminary one to three passages in milk at 45-50°C.

It should also be noted that when the lactic acid bacilli were isolated they did not produce a surface growth (an effect typical for the majority of freshly isolated *Lactobacterium* cultures).

Our work enabled us to settle on two main isolation methods. The choice of method depends on the degree of contamination of the sample by lactic acid bacilli.

Method 1. Low degree of contamination (raw and pasteurized milk, cream, washings from equipment): a) incubation of samples at 45-50°C for 16-18 hr; b) microscopic examination of sample; c) when large numbers of nonsporing bacilli are discovered the samples are plated out on agar containing hydrolyzed milk and colonies containing bacilli are subsequently isolated in sterile milk; d) if incubation of the sample at 45-50°C for 16-18 hr fails to produce a considerable enrichment with bacilli, the culture is passed another one to three times through sterile milk at the same temperatures.

Method 2. High degree of contamination (cottage cheese, sour creams, contaminated starter: a) microscopic examination of sample; b) if it contains a large quantity of bacilliform microflora it is plated out direct-

ly on agar containing hydrolyzed milk and typical colonies containing bacilli are then isolated in sterile milk; c) if direct plating out on agar fails to isolate the lactic acid bacilli, then, as in the first method, the sample is passed another one to three times through sterile milk at temperatures 45-50°C.

Study of Properties of Isolated Lactic Acid Bacilli

METHOD

As objects of study we took 50 isolated cultures of lactic acid bacilli: six strains of *Lactobacillus bulgaricus* (*Lactobacterium bulgaricum*) and five strains of *Lactobacillus casei-helveticus* (*Lactobacterium casei-helvicum*) (cheese bacillus), which were kindly given to us by the Pure Culture Laboratory of the All-Union Institute of Microbiology; two strains of *Lactobacillus acidophilus* (*Lactobacterium acidophilum*) and one strain of *Lactobacillus helveticus* (*Lactobacterium helveticum*), obtained from Kiev (UMIL); and two strains of *Lactobacillus acidophilus* (*Lactobacterium acidophilum*), which we had isolated in 1952-1953 from the excrement of farm animals.

All the strains were cultured in sterile milk.

We investigated the morphological, cultural, and biochemical characteristics of the cultures.

RESULTS

From a study of the isolated cultures we can give the following characterization.

Cells bacilliform, 4-10 × 0.7-0.9 μ, solitary, often with pronounced granularity. Gram-positive, nonsporulating, nonmotile. Microaerophilic.

Growth in milk and on agar containing hydrolyzed milk. No growth on meat-peptone agar. Submerged colonies, dark, yellowish-brown, sometimes with short diverging threads. In surface growth the colonies are large, curl-like, or granular, with a dark center.

Milk inoculated with one loopful coagulates within 8-10 hr, forming 1.5 to 2.2% lactic acid. Clot nonslimy or slimy, smooth, no gas. Casein not decomposed. Small quantity of volatile acids formed in milk.

Glucose, galactose, lactose, sucrose, maltose, levulose, raffinose, and dextrin are fermented with formation of acid.

Develop at high temperature.

Withstand brief heating in milk to 85-90°C.

Resistant toward common salt (up to 2-3%) and bile (up to 30-40%).

Some strains distinguished by considerable antibiotic activity, particularly against coliform bacilli and lactic acid streptococci (*Streptococcus lactis* and *Streptococcus thermophilus*, particularly the latter). Antibiotic effect of bacteriostatic type.

A comparative characterization of our isolated cultures and laboratory cultures of *L. bulgaricus*, *L. acidophilus*, *L. helveticus*, and *L. casei-helveticus* (cheese bacillus) is given in Table 1. It is clear from this char-

TABLE 1. Biochemical Characterization of Isolated Microorganisms and Stock Strains

Indices \ Name of species		Isolated micro-organisms	L.bul-garicus	L.acido-philus	L.casei-helvet-icus	L.helvet-icus
Time for coagulation of milk on inoculation by one loopful, in hr		8-11	8-11	8-11	8-11	8
Maximal acidity in milk, % lactic acid		1.5-2.2	2-3	2-3	1.9-2.8	2.5-2.7
Temperature limits for growth in milk	Minimal	20	20	20	25	25
	Optimal	45-55	45-50	40-45	40-45	45-50
	Maximal	60-65	62	60-62	60-62	60-62
	Lethal	85-92	75-80	70-80	75-80	78-80
Carbohydrate fermentation						
glucose		+	2+	+	+	+
galactose		±	-	+	+	
lactose		+	+	+	+	+
sucrose		±	±	-	-	+
maltose		+	-	-	+	+
levulose		+	-	-	+	-
raffinose		+	-	-	±	+
arabinose		-	-	-	-	-
mannitol		-	-	-	-	-
dulcitol		-	-	-	-	-
sorbitol		-	-	-	±	+
dextrin		±	±	-	±	+
Growth on hydrolyzed milk with NaCl in %		2-3	1	2	2-3	no growth
Growth on hydrolyzed milk with bile in %		30-40	no growth	20	10	"
Antibiotic activity (growth of <i>S. thermophilus</i> in dilutions of filtrate)						
Filtrate		-	-	-	-	-
1:2		-	±	-	-	+
1:4		+	+	±	±	±
1:8		±	+	±	+	+

Note: ± means some strains +, some strains -.

acterization that the isolated cultures must definitely be assigned to the lactobacillus (*Lactobacterium*) group, but they differ from the identified laboratory strains studied, and also from strains described in the literature (Lehmann and Neumann [1927]; Korolev [1940]; Krasil'nikov [1949]; Bergey [1923-1948]; Sharpe and Mattick [1957], and others) by their resistance to high temperatures and to bile.

As regards their ability to ferment carbohydrates the isolated heat-resistant bacilli are closest to the *L. helveticus* group and the cheese bacillus *L. casei-helveticus*. At the same time they possess antibiotic activity on a level with the antibiotic activity of the best industrially valuable strains of *L. acidophilus* (Koroleva [1960]; Polonskaya [1949]).

Further investigations will probably enable us to determine if the high resistance of the isolated lactic acid bacilli to heating and to high concentrations of bile is a species feature or a result of adaptation. In this connection a discovery of the causes of the increased heat resistance of these microorganisms would be of great interest.

Occurrence of Heat-Resistant Lactic Acid Bacilli in Raw Milk, Products, and on Equipment. Main Conditions Leading to Their Increase in Industrial Conditions

METHOD

To reveal the heat-resistant lactic acid bacilli in raw and pasteurized milk and in cream, we collected samples in a sterile vessel and incubated them at 45-50°C for 16-18 hr. The obtained cultures were then examined under the microscope. In the finished products - starter, cottage cheese, choice sour cream - we determined the presence of heat-resistant bacilli by direct microscopy of the preparations. At the same time we determined the Turner acidity of the products. We inspected the equipment by wiping it with a sterile cotton wad, after which the wad was put into sterile milk. The milk was incubated at 45-50°C for 16 hr and the obtained cultures examined under the microscope. From several samples of milk, finished products, and washings from the equipment taken during the manufacturing process, we isolated the lactic acid bacilli and subsequently identified them and ascertained their heat resistance.

TABLE 2. Occurrence of Heat-Resistant Bacilli in Sour Milk Products and Starters

Name of product	Number of specimens examined			
	total	contain- ing only strepto- coccal micro- flora	containing heat- resistant bacilli also	
			total	as % of total number
Cottage cheese	82	12	70	87
Starter for cottage cheese	2072	1698	374	18
Choice sour cream	311	154	157	50
<i>S. lactis</i> starter for sour cream	373	355	18	5
<i>S. thermophilus</i> starter for choice sour cream	337	328	9	3

Note: In compiling the table we also used the data obtained by the laboratory from the systematic inspection of starters and products over the past three and a half years.

We conducted investigations during the course of manufacture of cottage cheese and choice sour cream. We also examined samples of raw milk coming into the combine (from a milk tank truck); samples of milk pasteurized by holding at 75°C for 15-20 sec in appliances made by Alfa-Laval and APV; pasteurized milk heated again (for kefir) at 80°C for 15-20 sec in the Alfa-Laval appliance; cream (fat content 10%) pasteurized at 86-87°C; imported cottage cheese made from pasteurized and raw milk.

RESULTS

The data of Table 2, which gives the results of the microscopic examination of sour milk products and starters, indicate the widespread occurrence of heat-resistant lactic acid bacilli in these products.

For instance, 3-5% of the starters for choice sour cream and up to 18% of the starters for cottage cheese were rejected by the laboratory owing to their content of bacilli. We found heat-resistant bacilli in 50% of the samples of choice sour cream and in almost all the samples of cottage cheese. Representatives of this group were also found in raw and pasteurized milk, on equipment and pipes prepared for the reception of milk and cream (fat content 10% and 44%), and also in imported cottage cheese made from raw and pasteurized milk.

It was much more difficult to reveal heat-resistant bacilli in milk pasteurized at 75°C than in milk pasteurized at 80°C and 85°C. This can probably be explained by the fact that at comparatively low pasteurization temperatures the milk still contains a large percentage of thermophilic streptococci, which on subsequent incubation of the milk samples multiply rapidly and suppress the development of the few heat-resistant bacilli. Higher pasteurization temperatures destroy the majority of the heat-resistant streptococci with the result that more congenial conditions for the development of heat-resistant bacilli are created.

The greatest enrichment of products with heat-resistant lactic acid bacilli occurred, as we might have expected, in those products where the manufacturing process entails treatment at temperatures above 28-30°C. A rapid development of these organisms in choice sour cream was observed when the pasteurized cream was collected at 45-60°C before homogenization. During the souring of the cream at 45-47°C the heat-resistant bacilli continued to develop, despite the addition of large doses of streptococcal starter. In the manufacture of cottage cheese a rapid development of the bacilli occurred when the milk, and then the curd, was kept in the cheese vats at temperatures around 28-35°C. The appearance of lactic acid bacilli in starters was noted particularly frequently in cases where the normal process of development of the streptococci had been suppressed by some factor or another (bacteriophage, mastitis milk, autumn-spring milk), or when the pasteurization conditions were altered.

In kefir, which is soured at low temperatures (18-20°C), hardly any heat-resistant bacilli were found. However, in samples of kefir which had been soured at 37-40°C, these bacilli formed 60-70% of the microflora.

The obtained data indicate that the most important factors in the enrichment of sour milk products with heat-resistant representatives of the *Lactobacterium* group are the temperature conditions and the duration of the manufacturing process.

Parallel determinations of acidity and microscopic investigations of finished products invariably showed that products with acidity exceeding that permitted by GOST were generally heavily contaminated with heat-resistant bacilli.

The discovery of the microbiological causes of the excessive increase in acidity of milk products enables us to make certain recommendations to the industry for the prevention of this defect.

Pasteurization of milk and cream at high temperatures (90-95°C) for 20-30 min is effective in destroying the heat-resistant bacilli. However, the employment of high pasteurization temperatures in the production of sour milk products is hardly advisable, since this would lead to considerable physicochemical changes in the milk, thus reducing its biological value and entailing considerable extra expenditure of power on heating and cooling in the treatment of the milk. Prolonged pasteurization at high temperatures must be employed in the production of starters. It seems to us that work aimed at finding ways of checking the excessive rise of acidity in milk products during manufacture must be conducted along the following lines:

- 1) Reduction of duration of manufacturing process.
- 2) Reduction of souring temperatures where possible.
- 3) Rapid cooling of products after manufacture.
- 4) Selection of microflora for starters with reference, on one hand, to their antibiotic activity against heat-resistant lactic acid bacilli and, on the other, to their tolerance toward metabolic products of the heat-resistant bacillus.

SUMMARY

1. The excessive rise in acidity in sour milk products can be attributed to the development of a heat-resistant lactic acid bacillus, which is found in raw and pasteurized milk, in pasteurized cream, in starters, cottage cheese, choice sour cream and on the equipment.

2. Isolation of the heat-resistant bacillus by the usual methods used in the inspection of milk and milk products according to State standards is very difficult. The main conditions required for their successful isolation are preliminary enrichment of the milk, plating out on agar containing hydrolyzed milk, and cultivation at temperatures 45-50°C.

3. According to their morphological, cultural and biochemical characteristics, the isolated microorganisms can be assigned to the genus *Lactobacterium*. They differ, however, from the main thermophilic representatives of this genus, which have been described in the literature and which were investigated by us, in their high heat resistance in milk (growth unchecked up to 85-90°C) and their tolerance of 40% bile in the medium. As regards their ability to ferment carbohydrates, these microbes are most closely related to *Lactobacillus helveticus* (*Lactobacterium helveticum*) and the cheese bacillus *Lactobacillus casei-helveticus* (*Lactobacterium casei-*

helveticum) and in their antibiotic properties and tolerance of NaCl and bile are closest to *Lactobacillus acidophilus* (*Lactobacterium acidophilum*).

4. Pasteurization of milk and cream at 75, 80, 85-87°C in industrial conditions does not destroy the heat-resistant bacilli. Only pasteurization at 90-95°C for 20-30 min can ensure the total destruction of these microbes in milk.

5. The heat resistance of the investigated microorganisms is responsible for their rapid development in those sour milk products which are manufactured at temperatures above 28-30°C (cottage cheese, choice sour cream, *Streptococcus lactis* and *Streptococcus thermophilus* starters).

6. Excessive acidity of milk products can be prevented by a shortening of the manufacturing process and rapid cooling of the finished product. Another method of preventing this defect is the use of starters selected for their antibiotic activity and minimal sensitivity toward the heat-resistant bacillus.

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USE OF THE AUTORADIOGRAPHIC METHOD FOR A QUANTITATIVE ASSAY OF METHANE- OXIDIZING BACTERIA

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A quantitative assay of the methane-oxidizing bacteria in water and soil presents considerable difficulty. The well-known method suggested by Mogilevskii (1953) for microbiological prospecting of oil and gas deposits is the most widely employed. This method entails the grading of growth of a given group of bacteria according to a number system.

Quantitative assay by cultivation methods is unsatisfactory because the growth of a given group of bacteria in nutrient media frequently occurs only with a heavy inoculum. As a rule, growth will not proceed from a single cell. The assay method using Petri plates of selective, agar-containing medium in a mixed air-methane atmosphere is also unsuitable, since under these conditions a number of oligocarbophilic colonies appear on the surface of the agar. These are unable to oxidize methane but are supplied by that small quantity of soluble organic substances still present in the leached agar.

In this connection, we have devised a method based on autoradiography (Lomanenko 1959) for the investigation of the soil and water of the Rybinsk reservoir.

As a preliminary step, one to five ml of water were filtered through boiled No. 3 membrane filters. Next, Petri dishes were filled with a medium of the following composition (in grams): NaH_2PO_4 , 0.3; KNO_3 , 0.1; MgSO_4 , 0.05, leached agar 1.5; tap water 20 ml; distilled water 980 ml.

Before the medium is poured into Petri dishes one ml of radioactive sodium bicarbonate, $\text{NaHC}^{14}\text{O}_3$, with a specific activity of 0.2-0.3 μC is added.

In the assay of the methane-oxidizing bacteria in silt or soil, a 1:200 dilution of silt with sterile water is made. Several ml of sterile water are poured beforehand into a filtration funnel for more equal distribution of the bacteria and one or several drops of the culture is introduced. The filters are spread on the Petri dishes containing the medium described above.

The completed dishes are placed in a dessicator filled one-third with methane and two-thirds with air. In seven to ten days at 28-30°C microcolonies 0.1-1.0

mm in diameter grow out on the filters. While growing, methane-oxidizing bacteria assimilate the radioactive carbonic acid from the nutrient medium and their colonies become radioactive. The filter is then removed from the medium, dried on filter paper and treated with five per cent HCl to remove the radioactive carbonate which may have been deposited on the filter. This step may be accomplished in the Petri dishes; the acid solution is poured in and the filters are spread carefully with forceps on the surface of the fluid in order to avoid washing off the bacterial colonies. The HCl is then thoroughly washed out in plates containing distilled water. This treatment ought to be carried out under suction. After this the filters are dried a second time and are pasted on corrugated cardboard 10.5 × 3.5 cm. An ordinary Fedov photographic film of 65-90 units sensitivity, on which the filter number has been cut, is applied, emulsion side against the filter. The ends of the cardboard are turned down to press the film against the filter, and the latter is placed in a packet of black paper a little larger than the cardboard. Several such packets are put together under a press for four to five days. During this time the radioactive colonies of methane-oxidizing bacteria act on the photoemulsion. The film is subsequently removed and placed in contrast developer and fixative, and is carefully washed and dried. The imprints left by the microcolonies are counted under an MBC-1 binocular microscope. The count is performed in accordance with the amount of water filtered. A few filters may be used as controls by parallel incubation without methane. Saprophytes either do not show at all or leave a weak imprint after much longer exposure.

In studies using this method, water from the bottom layer of the Rybinsk reservoir contained from a few bacteria to a few hundred cells per milliliter and silt contained up to several tens of thousands of methane-oxidizing bacteria per gram. In simultaneous assay by the method of least dilution the number of bacteria found is 20-30 times less. I feel that the method described here can be fully applied to the counting of methane-oxidiz-

ing bacteria for investigative work in oil prospecting. It is quite possible that our method may also be used to count other groups of bacteria which oxidize gas-forming hydrocarbons.

SUMMARY

1. The described technique of assaying methane-oxidizing bacteria consists first of filtration of the material being tested through a membrane filter. The latter is then placed in a Petri dish on the surface of a mineral agar-containing medium to which carbon labeled sodium bicarbonate is added. Incubation is carried out for seven days in a methane atmosphere at 28-30 C.

2. After being treated with acid the filter is imposed on a photosensitive film. Autoradiographs of the colonies of methane-oxidizing bacteria are thus obtained.

3. This method proved 20-30 times more sensitive for assaying methane-oxidizing bacteria than the method of least dilution.

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POLAROGRAPHIC MODIFICATION OF THE BERTRAND METHOD FOR ASSAYING CARBOHYDRASE ACTIVITY

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The literature contains very little information on the use of the polarograph for the determination of enzyme activity. For instance, there is a reference to the polarographic determination of some oxidative enzymes in a paper by Knobloch [1943]. The aim of the present paper is to demonstrate another method of polarographic measurement of the activity of enzymes, particularly carbohydrases.

Several authors have dealt with the assay of sugars by means of the polarograph. For instance, Heyrovsky and Smöler [1932] and Canton and Penistow [1940] made direct determinations of sugar (glucose) by heating it to 60-70°C on a polarograph. This method is inconvenient, however, since mercury or calomel is used as the anode.

Grzhivo and Berkh [1959] also made direct determinations of sugar, but the absence of a comparison with the results obtained by the Bertrand method did not permit an assessment of the accuracy of the method.

Dlezek [1951] proposed an indirect method of determining reducing sugars by heating the glucose-containing solution with Fehling's reagent (I+II) for 5 min. A standard solution of glucose was added to one of the two test samples. When the cuprous oxide had been filtered off, the polarograms of the solutions were recorded and the difference in heights of the second wave of copper was subtracted from the control sample. The quantity of copper found was converted to sugar. It should be noted that the time spent on sugar determination by this method is no less than with the Bertrand method.

In our work we made an attempt to determine indirectly the quantity of reducing sugars by a method which combined the accuracy of the Bertrand method with the sensitivity of the polarographic method. In this case the quantitative assay of sugar after the test liquid containing the sugar had been heated by the Bertrand procedure was made from bivalent copper on the polarograph.

The incorporation of the polarographic method in the last stage of determination of sugars by the Bertrand method has certain advantages. The laborious process of filtration under vacuum is avoided, and hence the time spent on the sugar determination is reduced.

METHODS AND MATERIAL

Estimation of sugars by the polarograph reduces to a determination of the bivalent copper remaining after the test solution has been heated with Fehling's reagent. For this purpose we put into 100 ml flasks 10 ml of each solution of Fehling's reagent, and a volume of test solution depending on the sugar content of the sample. The total volume was brought to 30 ml with water. After the heating (3 min) and the precipitation of the cuprous oxide deposit, all the liquid was transferred to a 100 ml measuring flask and brought up to the mark with water. The solution was let stand for 30 min to ensure complete deposition of the cuprous oxide.

Incomplete deposition of the cuprous oxide affects the results of analysis. Hence, the supernatant liquid must be removed very carefully from the top by a pipette.

For polarography we took samples of the following composition: 1 ml of supernatant liquid, 1 ml of water and 18 ml of ammonia buffer containing 2 g NH_4Cl per 100 ml, 1.27 ml of NH_4OH (30% solution), 0.5 g Na_2SO_3 and 25 ml of 0.2% gelatin solution. The prepared mixture was put into the cell of the electrolyzer and the polarograms of bivalent copper were recorded.

The work was conducted on the electronic polarograph EP-212-TsLA . The current range was 20, the polarograph origin $E=0$ v, and a damping device was used to reduce the amplitude of the oscillations.

The characteristics of the capillary were $t=1$ sec and $m=2.41$ mg/sec. As a comparison electrode we used an external saturated Sochevanov calomel electrode. All the work was conducted in a thermostatically controlled cell at 25°C and height of mercury column 56 cm. The reduction current was determined from the height of the second wave of bivalent copper (h_2) by means of a standard curve.

For plotting the calibration curve we used a 4% solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (99% pure). Ten ml of this solution was heated for 3 min with 10 ml of the second Fehling reagent and 10 ml of water, and then the volume was brought to 100 ml with water. The samples for po-

TABLE 1. Comparative Determinations of Glucose by Bertrand Method and by Polarograph

Glucose quantities used, mg	Bertrand method				Polarography				
	KMnO ₄ , ml; T-6.3	copper, mg	glucose, mg	diff. from known glucose quant., mg	h ₂ , mm	h ₂ -h _s *	copper, mg	glucose, mg	diff. from known glucose quant., mg
0	—	—	—	—	67.5	—	—	—	—
4	1.5	9.45	4.73	+ 0.73	63.5	4	8.23	4.12	+ 0.12
8	2.7	17.01	8.56	+ 0.56	57.5	10	15.43	7.75	- 0.25
12	3.9	24.57	12.13	+ 0.13	51.5	16	23.65	11.65	- 0.35
16	5.2	32.76	16.28	+ 0.28	46.0	21.5	30.85	15.33	- 0.67
20	6.3	39.69	19.78	- 0.22	39.5	28	39.09	19.50	- 0.50

* h_s - height of Cu²⁺ wave in individual sugar samples.

larography consisted of 0.1 to 1.5 ml of the copper sulfate solution, 18 ml of ammonia buffer and enough water to bring the total volume to 20 ml. The obtained values for the height of the second wave of copper were plotted against the copper concentration in 100 ml (on basis of whole sample). From the height of the wave we deduct- ed 2 mm, the value of the residual current (Fig. 1).

Polarographic Determination of Glucose

In a series of experiments we used the polarographic method to determine known quantities of glucose.

We found a quantitative relationship between h₂ of the wave and the concentration of Cu²⁺ left after precipitation of the cuprous oxide (Fig. 2).

For measurement of the wave height we used the graphic method shown in Fig. 2, a. The difference in heights between the control and experimental samples was expressed as copper from the calibration curve. The quantity of sugar corresponding to the quantity of copper was found from Bertrand's table.

Since we had converted the Bertrand method to a polarographic determination of copper in the supernatant liquid we had to compare the results of glucose determination by the two methods. A comparison of these two methods was all the more necessary because we had

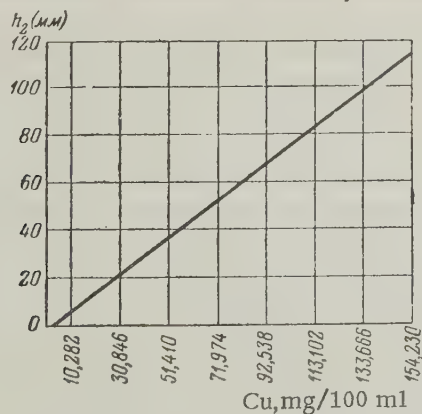


Fig. 1. Calibration curve of bivalent copper.

taken the supernatant liquid for the polarographic determination without filtering off the cuprous oxide.

The results are given in Table 1.

An analysis of the experimental data showed that the quantities of glucose determined by the Bertrand method slightly exceeded the true values, especially at low glucose concentrations. The polarographic method gave quantities of glucose close to the known quantities added. The lower values obtained in the polarographic determination indicate impurities in the glucose preparation.

Determination of Saccharifying Activity of Amylase of *Aspergillus oryzae* and *Bacillus subtilis*

In this work we used culture liquids, extracts of surface cultures, and also purified and crystalline preparations of amylase from *Aspergillus oryzae* and *Bacillus subtilis*.

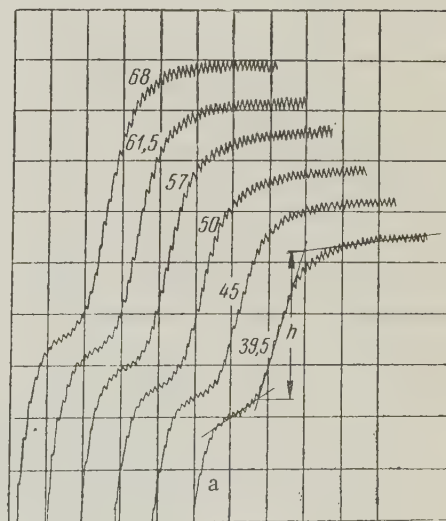


Fig. 2. Variation of height of Cu²⁺ wave with glucose concentration. a) Method of measuring wave height.

TABLE 2. Saccharifying Ability of Culture Liquid from *B. subtilis*

Method	h, mm	Maltose, mg	SA in mg per 100 ml of culture fluid
Polarographic	34.4	43.86	26316
Bertrand	-	43.43	26058

The saccharifying activity was determined from the ability of the amylase to produce maltose by action on starch. To 20 ml of a 2% starch solution we added the enzyme in appropriate dilution and enough water to bring the total volume of the mixture to 30 ml. The incubation temperature was 30 C, and the duration of incubation was 15 min. We added a 10 ml sample to Fehling's reagents, heated the mixture, and then diluted it to 100 ml, from which we took 1 ml for polarography. For an estimate of the preformed sugar (sugar formed before the action of the enzyme on the starch) the sample consisted of corresponding quantities of starch and enzyme, which were added separately to the mixture of Fehling's reagents. The copper waves obtained on the polarograph showed quantitative differences, depending on the action of the enzyme (Fig. 3).

The enzyme activity, expressed as the height of the copper wave (h_a), was found from the formula: $h_a = h_{pr} - h_{ex}$, where h_{pr} is the height of the sample wave due to preformed sugar and h_{ex} is the height of the wave of the experimental sample. The quantity of sugar and copper corresponding to h_a (in mm) was found from the standard

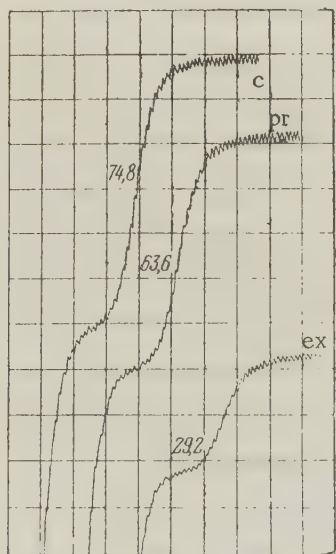


Fig. 3. Polarographic determination of saccharifying activity in culture fluid from *B. subtilis*.

curve and Bertrand's tables. When we needed to know the quantity of preformed sugar the sample consisted of Fehling's reagents (c).

Since the assay of the enzyme activity was reduced to a determination of the formed sugar (maltose) by Bertrand's method, the formula for calculating the saccharifying ability (SA) of the enzyme remained unchanged:

$$SA = \frac{\text{ml maltose} \times 30 \times 60}{C \times 15 \times 10},$$

where C is the quantity (ml) of enzyme solution taken for determination; 30 is the volume of the mixture; 60 is the conversion factor for 1 hr; 15 is the time of hydrolysis (in min); 10 is the sample (ml) of mixture for the determination of maltose.

To shorten the time of treatment of the results we could also use a simple estimate of the wave height (difference in heights of control and experimental samples) by taking an increase in height (h_a) of 1 mm as the polarographic unit of enzyme activity (by analogy with photometric determination of activity of some enzymes, where a change in extinction by one division is taken as the unit of activity).

Using purified preparations and culture liquids we compared the enzyme activities measured by the Bertrand method and by polarographic analysis (Table 2).

This showed that metal ions and other impurities in the media in which *A. oryzae* and *B. subtilis* had been grown had no effect on the height of the Cu^{2+} wave.

SUMMARY

1. A polarographic modification of the Bertrand method is proposed for the assay of reducing sugars. The method is based on a determination of the height of the polarographic wave of bivalent copper in the supernatant liquid after the Fehling reagents and sugar had been heated together.

2. A comparison of the results obtained by the Bertrand method and its polarographic modification showed that at low sugar concentrations the polarographic method gives a more accurate determination of the sugar content than the original Bertrand method.

3. The polarographic method can be used for the assay of the activity of some carbohydrases which act on starch, dextrans and disaccharides.

A 1 mm increase in height of the copper wave can be used as the polarographic unit of enzyme activity. This considerably shortens the time of analysis.

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PRESERVATION OF THE PROPERTIES OF WINE YEASTS BY THE FREEZE-DRYING METHOD

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When yeast cultures are maintained on the defined media used in laboratories, their activity and their ability to resist foreign microflora are often decreased. Scientists must therefore develop and use special methods to retard degeneration of the cultures.

The methods for preserving industrial cultures in collection laboratories are unsatisfactory and in need of drastic revision; Kudryavtsev [1951, 1958] has therefore recommended a method for the continuous selection of microbes from industry, by which the microbes would be maintained on artificial media for a minimal time, and by which the development of varieties more adaptable to the industry could be encouraged.

In order to prevent the degeneration of cultures in collection laboratories, Veselov [1951] recommended that the causes of variation be investigated. He proposed that microbes be preserved in collection laboratories maintained solely for specific industries.

Feniksova [1951] mentioned the methods of preserving the basic properties of cultures in laboratories — passage through liquid media, cultivation of yeasts from spores, frequent transfers, maintenance under anaerobic conditions, drying the cultures, and changing the maintenance temperatures. Bogdanov [1951] recommended vacuum drying during freezing (freeze-drying). This method is already in use in collection laboratories, in the production of bacteriophages, vaccines, and serums [Titov, 1945; Kolesov, 1952, 1959], and in the laboratories of industries which depend on the vital activities of microorganisms. Nevertheless, many problems connected with this method of preservation have not been sufficiently studied.

The purpose of this work was to study the problems involved in preserving the characteristics of wine yeasts by the method of simultaneous freezing and vacuum drying, and to determine the optimal conditions.

Wine yeasts *Saccharomyces vini* were used for study. The data obtained from the industrial strains "Apple 7" (2 strains) and "Cider 101" are presented.

The yeasts were cultivated on apple juice and unfermented beer wort. The yeasts were dried by a vacuum apparatus. A 48-hr yeast culture which had been grown on mats with beerwort agar was washed with a fluid of

the following composition: sucrose—10%, gelatin—1.5%, agar—0.1%.

The wash-off was diluted to a concentration of 10–12 billion yeast cells per ml of suspension. The concentration of living yeast cells in the source suspension was more accurately determined by the plate count method, using wort agar.

Samples of the culture (0.2 ml) were measured into ampoules. The ampoules were then immersed in dry ice, where their contents froze rapidly. The culture was maintained in the frozen state at normal atmospheric pressure for 0.5 hr. Then the ampoules were connected to the vacuum apparatus, which was already in operation with a residual pressure of 2–3 mm of mercury.

Drying and freezing were carried on simultaneously for the first 8–10 hr; the ampoules remained immersed in dry ice. Later the refrigerant was removed, and drying was continued at room temperature.

Granular calcium sulfate, which had been dehydrated by drying at 180–200°C, was used as a dehydrating agent.

The effect of the residual moisture on the preservation of dried yeasts was studied, in order to ascertain the optimal conditions of preservation. For this reason, drying was carried out for 16, 18, 20, 22, and 24 hr.

Periodically, at the designated times, 3–6 ampoules were detached by sealing under vacuum. One ampoule was used to determine the viability and properties of the dried yeasts. The second was used to determine the residual moisture content, and the other ampoules were retained for further observation. Altogether, 19 series of drying experiments were conducted. The residual moisture contents from all of the drying experiments ranged from 0.08–7.80%. The samples which had been dried for 18 hr by the method described contained about 2% of residual moisture. The residual moisture content was determined by drying at 105°C to constant weight. The viability and properties of the yeasts were studied immediately after drying and 3, 6, 8, 10, and 12 months later.

In order to determine the viability of the dried yeast, 0.5 ml of physiological saline solution was added to the yeast in the ampoule and allowed to stand for 1 hr at

TABLE 1. Relation of the Residual Moisture Content to the Viability of Yeasts Immediately after Drying

Yeast	Group	No. of exp.	Residual moisture, %	Viability, %
"Apple 7"	1	18	0.08—1.00	0.413
	2	17	1.06—1.50	3.350
	3	24	1.56—2.60	14.074
	4	12	2.80—4.00	0.930
	5	16	4.20—6.20	0.038
	6	8	6.50—7.80	0.006
"Cider 101"	1	18	0.09—0.90	0.840
	2	17	1.10—1.50	7.840
	3	24	1.52—2.63	32.090
	4	12	2.64—4.00	5.080
	5	16	4.30—5.13	0.851
	6	8	6.50—7.50	0.037

room temperature. A 0.1 ml sample of a determined dilution of the culture was measured into a sterile Petri dish and flooded with cooled wort agar. The plates were incubated at 28C and the colonies were counted after two different periods of incubation.

The multiplication rates and fermentation characteristics of the yeasts were used as the criteria of their physiological condition. The dried yeast cultures under observation were transferred to produce a second generation. The characteristics of the original cultures of the yeasts were studied in the same manner. In order to determine the multiplication rate, apple wort containing from 12.5 to 13.7% sugar was inoculated with a measured volume of the second-generation liquid culture. The number of cells was estimated in the Tom-Zeiss chamber at the time of inoculation and 24, 48, and 72 hr later.

The fermenting activity of the cultures was determined by the amount of CO₂ and alcohol produced and by the residual sugar content. For CO₂ determination, the yeasts were cultivated in liquid apple wort in flasks with Meissel traps. The alcohol content of the fermented wort was determined by the specific gravity of the distillation product. The residual sugar in the medium was determined by Bertrand's method.

RESULTS

The viability of wine yeasts dried under vacuum is closely connected with the amount of residual moisture. All of the dried cultures can be tentatively divided into six groups, according to their residual moisture content (Table 1).

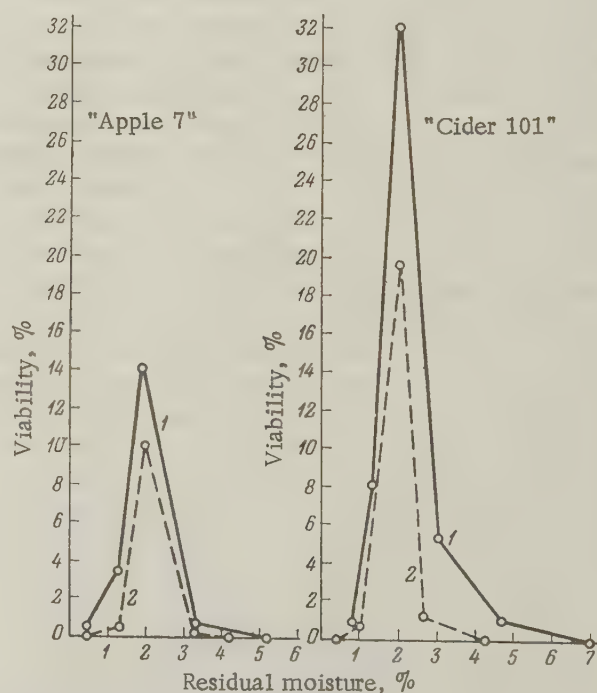
The residual moisture content and the viability of the yeasts immediately after drying are given in Table 1.

As shown in Table 1, the largest percent of viable cells was found in the third group immediately after drying; the residual moisture content of this group had been 1.52 to 2.63%.

TABLE 2. Relation of the Residual Moisture Content to the Multiplication Rate of Yeasts Immediately after Drying (1 million seeded)

Culture	Group	Residual moisture, %	Quant. of cells in 1 ml med. (mil.)		
			after 24 hr	after 48 hr	after 72 hr
Original "Apple 7"	—	—	5.8	8.4	9.8
	1	0.08—1.00	4.4	7.1	8.3
	2	1.06—1.50	4.3	7.4	10.2
	3	1.56—2.60	4.0	7.7	13.5
	4	2.80—4.00	3.0	6.2	8.1
	5	4.20—6.20	4.8	6.8	8.4
Dried	6	6.50—7.80	4.8	6.7	6.4
Original "Cider 101"	—	—	8.1	12.5	14.3
	1	0.09—0.90	6.5	8.3	9.1
	2	1.10—1.50	6.8	9.1	15.1
	3	1.52—2.63	7.2	9.4	16.2
	4	2.75	6.2	9.6	10.3
	5	4.30—4.70	6.2	7.3	9.7
Dried	6	6.50	6.7	7.7	9.9

Later observations showed that the viability of the third group (residual moisture—1.52-2.63%) remained highest during maintenance for one year. After twelve months of maintenance, the viability of cultures in this group decreased (in "Apple 7" to 10.1% and in "Cider 101" to 19.04%); in the second group (residual moisture content 1.06-1.50%), the viability dropped to 0.038% in "Apple 7" and to 0.61% in "Cider 101"; in the fourth



The relation of the residual moisture content to the viability of yeasts. 1) Viability immediately after drying; 2) viability after one day of maintenance.

group (residual moisture content to 4%), "Apple 7" dropped to 0.87% and "Cider 101" to 0.37%. In the first group (moisture content to 1%), and the fifth group (moisture content to 6.2%), no viable cells were found (figure).

After the first two periods of incubation, the second and third groups of yeasts had a lower multiplication rate than the control yeasts; after 72 hr, however, they had a higher rate than the control yeasts. The yeasts of the remaining groups lagged behind the control yeasts in rate of multiplication (Table 2).

TABLE 3. Comparative Activity of the Fermentation of Apple Wort by the Original and the Dried Cultures Immediately after Drying.

Yeast	Group	Residual moisture, %	CO ₂ loss in 30 ml med. g	Residual sugar, %	Alcohol, vol. %
Original "Apple 7"	—	—	1.60	0.09	8.75
Dried	1	0.08—1.00	0.95	0.21	5.50
	2	1.06—1.50	1.22	0.11	7.80
	3	1.56—2.60	1.66	0.09	8.89
	4	2.80—4.00	1.07	0.12	7.75
	5	4.20—6.20	0.95	0.18	5.75
	6	6.50—7.80	0.89	0.21	5.20
Original "Cider 101"	—	—	1.90	0.06	8.98
Dried	1	0.09—0.90	1.64	0.09	8.76
	2	1.10—1.50	1.68	0.07	8.93
	3	1.52—2.63	1.82	0.07	9.14
	4	2.75	1.80	0.07	9.06
	5	4.30—4.70	1.52	0.14	8.14
	6	6.50	1.52	0.15	7.18

The multiplication rates of dried yeasts followed the same pattern during maintenance.

Relation of the Residual Moisture Content to the Fermenting Activity of Dried Yeasts

The fermentation activities of both the original and the dried yeast cultures were determined in the same manner, using an equal amount of cells from each. The yeasts were inoculated into apple wort containing 13.5% sugar.

Table 3 shows the data obtained from the determinations of CO₂, alcohol, and residual sugar after fermentation of wort.

From Table 3, it is apparent that the maximal amount of alcohol and carbon dioxide and the minimal amount of residual sugar were found after fermentation by the third group. These values are closer than the others to the magnitudes which characterized the original cultures.

Table 4 shows the data obtained from fermentation by the dried yeasts after 3, 6, 8, 10, and 12 months.

From Table 4, it is evident that group three had by far the most stable fermentation properties. Yeasts which had been preserved with other proportions of residual moisture showed a decrease in alcohol production. There was no evidence of fermentation by the fifth group after 10 months, nor by the first group after 12 months.

SUMMARY

1. Freezing with vacuum drying to a determined residual moisture content is a promising method for stabilizing the properties of wine yeasts.

TABLE 4. Comparative Activity of the Fermentation of Apple Wort (according to alcohol production) by Yeasts after Maintenance in Dried Condition for 3-12 Months.

Culture	Group	Residual moisture, %	immediately after drying	Alcohol, vol. %				
				time of maintenance, months				
				3	6	8	10	12
"Apple 7"	1	0.08—1.00	5.50	5.21	5.25	4.12	3.52	No fermentation
	2	1.06—1.50	7.90	7.80	7.88	6.75	6.25	6.00
	3	1.56—2.60	8.89	9.92	9.52	9.22	9.28	9.31
	4	2.80—4.00	7.75	7.67	6.32	4.10	3.87	3.46
	5	4.20—6.20	5.75	4.93	2.09	2.05	No fermentation	No fermentation
	6	6.50—7.80	5.26	4.09	2.00	1.91	—	—
"Cider 101"	1	0.09—0.90	8.76	7.92	7.27	7.01	5.72	No fermentation
	2	1.10—1.50	8.93	8.55	8.90	9.07	7.78	0.09
	3	1.52—2.63	9.14	9.03	9.25	9.15	9.35	9.08
	4	2.75	9.06	5.33	4.31	4.00	4.05	3.17
	5	4.30—4.70	8.14	5.50	4.09	3.77	No fermentation	No fermentation
	6	6.50	7.18	4.00	3.71	—*	—	—

* — Indicates no determination made.

2. The most favorable results were obtained when the residual moisture content was within the limits of 1.56-2.60%. Dried yeasts which contained this proportion of moisture preserved their original properties for 12 months.

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GREEN SULFUR BACTERIA

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With the exception of a few representatives the microorganisms known under the name of green sulfur bacteria have so far received little study. Yet there is no doubt that these microorganisms, like the purple bacteria, are of definite interest in research on the photosynthetic process and on the sulfur cycle in nature.

The term green bacteria appeared in the literature in about 1880. But there is some evidence that green microorganisms of bacterial nature were described even earlier, by Perty [1852] and Rey-Lankaster [1873].

In 1880 a paper was published by van Tieghem on two nonmotile green organisms, which he called Bacterium viride and Bacillus virens.

Shortly after, Engelmann [1882], who was studying bacterial phototaxis, discovered in Spirillum preparations a motile green organism, which he called Bacterium chlorinum.

When these bacteria were illuminated in an environment deficient in oxygen they formed concentrations in certain regions of the spectrum. Engelmann believed that Bacterium chlorinum contained chlorophyll and carried out photosynthesis of the same type as green plants, i.e., with the evolution of oxygen.

The same conclusion was reached by Winogradsky [1888], who turned his attention on the fact that the development of purple bacteria was often accompanied by the appearance in the same spots of very tiny green microorganisms in the shape of cocci, rods, and filaments. Winogradsky called them green bacteria and advanced the hypothesis that these microorganisms supplied oxygen to the purple bacteria, thus ensuring the development of the latter in conditions of air deficiency.

In subsequent years there appeared a considerable number of works containing descriptions of the various green microorganisms which often occur in polluted ponds, lakes, sulfur springs and other waters containing hydrogen sulfide, i.e., in places where purple bacteria usually grew (Dangeard [1890, 1895, 1909], Ewart [1897a,b], Nadson [1906, 1912], Lauterborn [1906, 1913, 1915], Streszewski [1913], Buder [1913], Skene [1914], Perfil'ev [1914a,b,c], Metzner [1922], Baas-Becking [1925], and Ponomarev [1929]).

In view of their morphological features, and sometimes also of their mode of life, which resembled that

of purple bacteria, they were separated from the green and blue-green algae and were called green bacteria.

The most interesting of the listed investigations are the works of Ewart [1897a, b], who described several microorganisms occurring in sapropel. Among them were the green Spirillum, which had been mentioned earlier by Rey-Lankaster [1873] and was later described by Benecke [1912], and a green microbe, which Ewart called Streptococcus varians.

According to the descriptions, the latter organism is identical with the green sulfur bacteria, which were thoroughly studied by Nadson [1906, 1912] and which he called Chlorobium limicola.

Almost simultaneously with Nadson's first communication on Chlorobium limicola there appeared Lauterborn's work [1906] with a description of Chlorochromatium aggregatum. Lauterborn established that Chlorochromatium aggregatum consisted of rod-shaped outer green cells surrounding a colorless central part. A microorganism similar to Chlorochromatium aggregatum was studied in more detail by Buder [1913], who gave it the name Chloronium mirabile. In Buder's opinion, Chloronium mirabile is identical not only with Chlorochromatium aggregatum Lauterborn (1906) but possibly also with Bacterium chlorinum Engelmann [1892], and Bacillus virescens Dangeard (1909).

The existence of symbiotic organisms, one of which is green in color, was confirmed later by several investigators (Perfil'ev [1914c], Isachenko [1927], Ponomarev [1929]).

Perfil'ev found Chlorochromatium aggregatum (Chloronium mirabile) in ponds situated near Leningrad. In addition, he described an independent species Cylindrogloea bacterifera, which, in contrast to Chlorochromatium aggregatum, was nonmotile and had a larger central cell.

The central component in Chlorochromatium aggregatum and in Cylindrogloea bacterifera is believed to be a colorless bacterium, whereas Chlorobacterium symbioticum (Lauterborn, 1915) represents a symbiosis of green bacteria with some protozoan.

Even before the description of Chlorobacterium, Lauterborn [1913] made an attempt to classify the "green bacteria." He separated them into a family Chlorobac-

teriaceae, in the belief that all its representatives were similar to the purple bacteria as regards nature of pigments and mode of life. Lauterborn included four genera in the family Chlorobacteriaceae: the symbiotic Chlorochromatium and the free-living Pelodyction, Schmiedlea, and Peloglea.

The bacteria assigned to the last two genera had previously been described by other authors as different species of Aphanothece (Schmidle [1901], West and West [1906], Szafer [1910]).

The morphology and conditions of development of Pelodyction were studied later by Perfil'ev [1914a,b], who found an abundant growth of these organisms in ponds in St. Petersburg Botanical Garden. Under laboratory conditions Pelodyction grew in the light in anaerobic culture in jars containing water and mud. According to Perfil'ev's data, Pelodyction cells are nonmotile and are rod-shaped or round. Their size is $0.5-1 \times 1-4 \mu$. The cells either form irregular aggregations, or are arranged in parallel rows, or produce fairly regular reticulate colonies.

A further step in the classification of green bacteria was the work of Geitler and Pascher [1925], who suggested calling these organisms Cyanochloridinae-Chlorobacteriaceae, thus stressing the close relationship between the green bacteria and the blue-green algae. In characterizing the family which they had erected, Geitler and Pascher indicated that it brought together unicellular bacterium-like organisms of various shapes and of a green or yellowish-green color.

On the basis of differences in the shape of the cells and the colonies formed by them, the representatives of the family Cyanochloridinae-Chlorobacteriaceae were divided into ten genera. Eight of these genera — Sorochloris, Tetrachloris, Chloronostoc, Schmiedlea, Pediochloris, Clatrochloris, Pelodyction, and Peloglea — contained the free-living species. The last two genera were represented by the symbiotic organisms Chlorochromatium and Chlorobacterium.

Geitler and Pascher's work terminated a definite period in the study of green bacteria. It can be described as the descriptive period, the most interesting feature of which were the discoveries of new species of microorganisms living in deep water containing hydrogen sulfide.

Beside giving a description of the shape of the cells and the colonies formed by them, many authors have attempted also to discover the nature of the cell pigments by determining the absorption spectrum and making some chemical tests. These studies, beginning with those of van Tieghem [1880] and Engelmann [1882], repeatedly revealed that the pigments of various microorganisms classed as green bacteria were very similar to plant chlorophyll (Ewart [1897b], Dangeard [1909], Perfil'ev [1914c], Monteverde and Perfil'ev [1914]). Some authors even considered it possible to claim that they were identical (Nadson [1912], Buder [1913]). Lauterborn [1913] called

the pigment of representatives of the Chlorobacteriaceae bacteriochlorin, i.e., the same name as was given to the pigment of purple bacteria at that time (Molisch [1907]).

That "the pigment of green microbes, despite its optical similarity, differs from typical chlorophyll and is more closely related in its properties to bacteriochlorin" was also indicated by Lyubimenko [1924, 1935], who called it prochlorophyll, and by Ponomarev later [1930].

Metzner [1922], however, came to the conclusion that the absorption spectrum of the pigment of green bacteria exhibited differences from the chlorophyll of plants and the chlorophyll of purple bacteria. Hence, he suggested that it should be called bacterioviridin. At the same time, Metzner noted that in chemical nature bacterioviridin was probably close to chlorophyll and, like it, could serve as an intermediary in the assimilation of carbon dioxide.

In other words, Metzner, like the majority of other investigators (Engelmann [1882], Winogradsky [1888], Buder [1913], Skene [1914]) suggested that green bacteria were capable of photosynthesis. Some authors had even noted that the evolution of oxygen in the presence of light could easily be detected in the green microorganisms which they studied (Engelmann [1882], Ewart [1897a, b], Buder [1913]).

At that time this fact was regarded as proof of photosynthesis.

Nadson [1912], however, who studied the conditions of growth of Chlorobium, and later Perfil'ev [1914], who had obtained enriched cultures of Pelodyction, could not detect evolution of oxygen by these organisms in the light. Hence, these authors cast doubt on the ability of green bacteria to accomplish photosynthesis and they wrote that Chlorobium and Pelodyction contained a "non-functioning chlorophyll."

Thus, the question of the nature of the metabolism of green bacteria and, in essence, the question of their separation into an independent taxonomic group remained open. The solution of this question, like the solution of that of the metabolism of purple bacteria, was due to the investigations of van Niel.

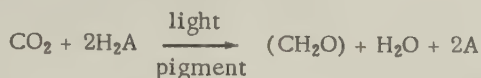
Having isolated Chlorobium limicola in pure culture for the first time and conducting his experiments in strictly controlled conditions, van Niel [1931] established that these organisms developed by a distinctive type of photosynthesis. In contrast to green plants, they grow and reduce carbon dioxide only in anaerobic conditions and in a medium containing hydrogen sulfide, which they oxidize to free sulfur.

A determination of the quantitative relations between the carbon dioxide consumed by the green bacteria and the oxidized hydrogen sulfide showed that the results of these processes could be expressed approximately by the following equation:



Simultaneously with his studies of the green bacteria, which were becoming correctly designated as green sulfur bacteria (or yellow-green sulfur bacteria), van Niel performed similar experiments with various representatives of the purple sulfur bacteria.

Comparing the results obtained in a study of the photosynthetic process in purple and green bacteria with the photosyntheses of plants, van Niel deduced a generalized equation for photosynthesis:



After van Niel's studies great interest was shown by research workers in the photosynthetic bacteria as organisms suitable for the study of the mechanism of photosynthesis and a comparison of it with the photosynthesis of green plants.

But so far, studies have been mainly devoted to the purple bacteria. The green bacteria have received much less study and only in recent years has some new information been obtained about these interesting organisms.

Until recently, the only representatives of the family Chlorobacteriaceae that had been isolated in pure culture and studied in controlled conditions were species of Chlorobium. Hence, the detailed characterization of the green sulfur bacteria (Bavendam [1936], Krasil'nikov [1949], Bisset [1954], Bourrelly [1954, 1955], Lees [1955], Bergey [1957]) is essentially a characterization of representatives of the genus Chlorobium. The correct assignment of other organisms to the green sulfur bacteria is a debatable question in a number of cases, since information about them is very limited.

van Niel [1931], and later Pringsheim [1949, 1953a, b] correctly pointed out that some of the microorganisms which Geitler and Pascher had included in the family Cyanochloridinae-Chlorobacteriaceae were small forms of algae, and some were identical with one another.

van Niel suggested that the green sulfur bacteria were all confined to the genus Chlorobium. However, the family Chlorobacteriaceae at present still retains six genera — Chlorobium, Pelodyction, Clatrochloris, Chlorochromatium, Chlorobacterium, and Cylindrogloea (Bergey [1957]).

The name Chlorobium has been extended to organisms previously assigned to the genera Pelogloea, Tetrachloris, Sorochloris, and Chloronostoc. In fact, if we refer to the classifications of Lauterborn and Geitler, the name Chlorobium does not occur in them. Yet these organisms are widely distributed in nature and they could hardly be found only by Ewart and Nadson.

It is obvious that Chlorobium species have been described several times under different names. This is all the more likely in view of the fact that, according to the data of several authors, Chlorobium is variable and the nature of their growth can vary considerably with the conditions of the medium (Nadson [1912], van Niel [1931]).

Judged from the descriptions, not only the organisms listed above, but also Winogradsky's green bacteria [1888] and the microorganisms discovered by Baas-Becking [1925] are typical Chlorobium, particularly since they frequently develop in association with purple bacteria. However, the view that Chlorobium and Chloropseudomonas are identical is not fully justified (Bergey [1948]). Although the latter organism has received little study, Czurda and Maresch [1937], who found it among purple bacteria, pointed out that Chloropseudomonas, in contrast to Chlorobium, possesses motility.

Another surprising feature is that many authors still include among the green sulfur bacteria consortia consisting of colorless and green organisms, such as Chlorochromatium, Chlorobacterium and Cylindrogloea (Bourrelly [1955]; Bergey [1957]).

In this respect Krasil'nikov [1949] is more correct in assigning only two genera — Chlorobium and Pelodyction — to the family Chlorobacteriaceae.

Mechner [1957] believes that the green sulfur bacteria should include Chlorobium, Microchloris, and perhaps Clatrochloris. In the genus Chlorobium he includes Pelodyction and the green sulfur bacteria growing in symbiosis with colorless organisms.

The sole representative of the genus Microchloris is M. nadsonii — a small rod-shaped organism discovered by Pringsheim [1953a]. The absorption spectrum of the pigments of M. nadsonii is the same as those of Chlorobium. This justified its inclusion in the family Chlorobacteriaceae.

All that is known about Clatrochloris sulphurica (Aphanothece sulphurica Szafer 1910) is that it grows in sulfur springs and contains sulfur droplets in the cell. These two features are not a complete guarantee that it belongs to the green sulfur bacteria.

Another obscure question is the nature of the so-called "green bacteria" which often appear in sewage water and the surface layers of other waters (Zakharov [1929], Mein [1932], Vinberg and Sivko [1952], Kuznetsov [1955]).

According to Godnev and Vinberg [1951], these rod-shaped aerobic organisms, called Bacterium chlorophyllorum (Vinberg and Sivko [1952]) contain the typical chlorophyll and carotenoids peculiar to green plants.

It has also been established that during photosynthesis B. chlorophyllorum produces oxygen. The above features exclude it from the green sulfur bacteria.

So, we turn now to the representatives of the genus Chlorobium, which beyond any doubt are not algae, but green sulfur bacteria.

As we already mentioned above, these organisms are of wide occurrence in nature.

The main habitats of Chlorobium are various waters containing hydrogen sulfide — ponds, lakes, sea bays, limans, and sulfur springs (Nadson [1906, 1912], Isachenko [1927], Pel'sh [1937], van Niel [1931], Jimbo [1937, 1938], Skuja [1948], Larsen [1953], Baas-Becking and Wood

[1955], Kaplan [1956]). These bacteria may be isolated from soil, although their numbers there are usually limited.

Under favorable conditions, i.e., in the presence of light and hydrogen sulfide, Chlorobium frequently forms large concentrations in the water and colors it green. Such concentrations are often observed in certain salt and fresh-water lakes (Manning and Juday [1941], Dutton and Juday [1944], Bicknell [1949], Newcombe [1950], Newcombe and Slater [1950], Kuznetsov [1952], Butlin [1953], Butlin and Postgate [1953a]).

Their development in the form of separate patches and zoogloea on the bottom of shallow waters has also been observed (Nadson [1912], Ponomarev [1929], Kaplan [1956]).

There is no doubt that when green sulfur bacteria develop in abundance along with other microorganisms they play an important role in the life of waters. They help to rid the waters of hydrogen sulfide (sometimes with the deposition of sulfur) and enrich them with organic matter (Butlin [1953], Butlin and Postgate [1953 a, b; 1954]).

It was not so long ago that only one species, C. limicola (Nadson [1906, 1912]), was assigned to the genus Chlorobium. This species, according to van Niel [1931], can grow only in a mineral medium containing hydrogen sulfide. In 1952, however, Larsen discovered green sulfur bacteria which differed from C. limicola in their ability to oxidize thiosulfate. In view of this character they were assigned to an independent species Chlorobium thiosulfatophilum.

In 1957 Mechsner, who was studying the symbiotic complex Chlorochromatium aggregatum, isolated its green components in pure culture. In their morphology, pigments, and a number of physiological features the isolated bacteria were closely related to the free-living species of Chlorobium. Hence, Mechsner called them Chlorobium chlorochromatii. That the green bacteria contained in Chlorochromatium were closely related to Chlorobium had been indicated earlier by several authors (Buder [1913], van Niel [1931], Pringsheim [1953a]).

In morphology the three species of Chlorobium are similar to one another. They are nonmotile, short rods with rounded ends, and sometimes the cells are almost round or resemble ellipses. Their size varies from 0.7-0.8 × 1-1.5 μ (Nadson [1906, 1912], van Niel [1931], Larsen [1952, 1953], Shaposhnikov et al. [1958]; Kondrat'eva, Fedorov and Greshnykh [1958], Skalinskii and Kondrat'eva [1961]).

The bacteria reproduce by transverse fission and do not form spores. Their cells are often grouped in chains of varying length and are surrounded by slime. It is of interest to note that when Chlorobium chlorochromatii is separated from the colorless bacterium it retains the ability for normal division and growth (Buder [1913], Perfil'ev [1914c], Mechsner [1957]).

According to Nadson [1906, 1912] and van Niel [1931], the shape of Chlorobium cells may vary with the

conditions of the medium. In addition to the streptococci and cells of ellipsoidal form, which Nadson and van Niel regarded as normal for C. limicola, they observed long rods, swollen round cells, and twisted forms in cultures of these bacteria. van Niel and Larsen [1953] believe that the main factors responsible for the appearance of involution forms are unfavorable pH and high concentration of hydrogen sulfide.

Possibly one such form was Chlorobium vibrioformis (Pel'sh [1937]). However, no one has studied this organism in pure culture.

Bicknell [1952], who succeeded in obtaining a pure culture of C. limicola from a single cell, noted that such cultures always contained uniformly shaped cells about 1 μ long.

As distinct from certain species of purple bacteria, all the Chlorobium species deposit the sulfur outside the cell when they oxidize hydrogen sulfide. This is easily detected from the characteristic clouding of the medium. Larsen [1953] noted that when C. limicola grew within agar a yellowish halo, consisting at first of sulfur droplets, and later of sulfur crystals, appeared around its discoidal colonies.

Around C. thiosulfatophilum colonies, which also have a discoidal or three-lobed form (Shaposhnikov [1958]), the appearance of such a halo is rarely observed.

Chlorobium species are gram-negative. The cell walls of C. thiosulfatophilum, like those of other bacteria, contain a number of sugars and amino acids. In particular, isomers of diaminopimelic acid, glucosamine, and the amino sugar 3-0- α -carboxyethylhexosamine have been found (Salton [1957]).

In the nucleotide composition of its RNA C. thiosulfatophilum resembles the purple bacteria (Chromatium) and the chemoautotroph Nitrosomonas. In the nucleotide composition of their DNA, however, these three bacteria differ from one another (Vanyushin and Belozerskii [1960]). Moreover, it has been noted that in DNA composition the investigated photosynthetic bacteria show a similarity with green algae, but differ distinctly from the higher plants. This is of great interest for an understanding of the evolution of photosynthetic organisms.

From this viewpoint, as well as for an understanding of the photosynthetic mechanism, a study of the pigment system of green sulfur bacteria is of great value (Elsden [1955], Stanier [1957, 1958]). It has been found that the cells of C. limicola and C. thiosulfatophilum contain definite structures in which their pigments are localized (Thomas [1950], Gibson [1957], Vatter and Wolfe [1957, 1958]. According to Vatter and Wolfe, the size of these particles, called chromatophores, in C. limicola is a little smaller than in purple bacteria and is 150-250 Å. Their role is probably comparable with that of the purple bacteria chromatophores, which have been shown to possess photochemical activity (Newton et al. [1957, 1958], Anderson [1958], Frenkel [1958, 1959], Frenkel and Hickman [1959]).

Living cells of green sulfur bacteria possess a characteristic absorption spectrum with the main maxima at 730-747 m μ and around 457 m μ in different strains (Katz and Wassink [1939], Larsen [1953], Shaposhnikov et al. [1958], Krasnovskii et al. [1959]).

By studying the absorption spectrum of the green pigment from pure cultures of *C. thiosulfatophilum*, and also of its pheophytin, Larsen [1953] confirmed Metzner's data [1922] which indicated that green bacteria contain a special chlorophyll, and he named it chlorobium chlorophyll.

However, Pringsheim [1953a,c], and later Seybold and Hirsch [1954], claimed that the chlorophyll of green sulfur bacteria was identical with the bacteriochlorophyll of purple bacteria, and that bacterioviridin was a product of its degradation. In this connection Goodwin [1955] made a thorough study of the green pigment of *C. thiosulfatophilum* by the use of column chromatography on sucrose. According to Goodwin's results, true chlorobium chlorophyll in sulfuric ether has absorption maxima at 659, 431, and 408 m μ . When homogeneous chlorobium chlorophyll, bacteriochlorophyll, and chlorophyll *a* were studied by chromatography on a column with sucrose these compounds were easily separated. A similar result was obtained in the chromatography of their pheophytins. Thus, Goodwin, and also several other research workers (Barer [1955], Mechner [1957], Krasnovskii et al. [1959, 1960], Kaplan and Silberman [1959]), confirmed that green sulfur bacteria contain a special chlorophyll, which at present still has two names — chlorobium chlorophyll and bacterioviridin. Depending on the solvent, the absorption maxima of this pigment may vary to some extent, but they are always shifted toward the short-wave side when compared with the maxima of intact cells. Such changes, as we know, also occur when plant chlorophylls or bacteriochlorophyll are isolated. According to Lyubimenko [1921] and several other authors (Katz and Wassink [1939], French [1938, 1940], van Niel [1944]), these changes are due to the fact that these pigments are bound with proteins in the cells.

From a study of the absorption and fluorescence spectra of *C. thiosulfatophilum* chlorophyll in various conditions, Krasnovskii et al. [1959, 1960] concluded that a considerable amount of this pigment, like bacteriochlorophyll (Krasnovskii and Voinovskaya [1952]), was present in an aggregated form in the cells (Krasnovskii [1957]). The light absorbed by this form is used for photosynthesis.

Before the studies of Krasnovskii and his colleagues, only scattered notes on the fluorescence of chlorobium chlorophyll were known (Seybold and Hirsch [1954], Williams [1956b], Goedheer [1958]).

Krasnovskii and Erokhin [1959] established that the main maxima in the fluorescence spectrum of living cells of *C. thiosulfatophilum* occurred at 670 and 740-750 m μ , but in a pyridine solution the fluorescence

maxima of chlorobium chlorophyll were observed at 675 and 752 m μ .

After a study mainly of the spectral properties of the chlorophyll of various strains of green sulfur bacteria, it appeared certain that all these organisms contained the same green pigment. Hence, the paper by Stanier and Smith [1960] indicating that two strains of *C. thiosulfatophilum* differed in their green pigment was rather unexpected. One of these strains contained the previously known chlorobium chlorophyll with absorption maxima in ether at 660, 432, and 412 m μ . In the other strain the absorption maxima of the chlorobium chlorophyll occurred at 650, 425, and 406 m μ . The absorption spectra of intact cells and the pheophytins, and the fluorescence spectra of the chlorophylls and also their absorption coefficients, were also different. Chlorobium chlorophyll 600 contained 2.95% Mg, whereas chlorobium chlorophyll 650 contained 2.59% Mg.

It is still not clear how these results should be interpreted. The authors themselves noted that chlorobium chlorophyll 650 was found only in one strain of *C. thiosulfatophilum*. The remaining seven investigated strains of green bacteria, which included *C. thiosulfatophilum* and *C. limicola*, contained chlorobium chlorophyll 660. Hence, even different species of *Chlorobium* could have the same green pigment.

In 1940 Fischer put forward the hypothesis that this pigment of green bacteria was 2-acetyl-chlorophyll *a*. However, from a study of the chemical properties of chlorobium chlorophyll 660 Holt et al. [1960] concluded that this pigment differed in several ways from all the known chlorophylls and certainly could not be 2-acetyl-chlorophyll *a*, since it gave no specific reactions.

There remains no doubt, however, that this pigment plays the main, if not the only, role in the assimilation of light energy by green sulfur bacteria. Larsen [1953] showed that the action spectrum of photosynthesis in *C. thiosulfatophilum* coincided with the absorption spectrum of chlorobium chlorophyll. In the presence of ascorbic acid or Na₂S, chlorobium chlorophyll, like chlorophyll *a*, is capable of reversible photoreduction. It is also capable in solution of sensitizing the photochemical transfer of hydrogen to the azo-dye methyl red (Krasnovskii and Pakshina [1959]).

Beside a special chlorophyll, green sulfur bacteria contain a small amount of carotenoids.

The first information about this was obtained by Perfil'ev and Monteverde [1914], who studied *Pelodyction* pigments, and then by Katz and Wassink [1939]. Larsen [1953] and Pringsheim [1953] also pointed out that *C. thiosulfatophilum* cells obviously contain carotenoids, which are responsible for the light absorption in the region 467-496 m μ . Barer [1955] suggested that green sulfur bacteria contain γ -carotene.

Goodwin and Land [1955, 1956], who employed chromatography of pigment extracts from *C. limicola* and *C.*

thiosulfatophilum after the chlorobium chlorophyll had been removed from them, confirmed that the main carotenoid (up to 80-85%) in these bacteria was γ -carotene. Beside γ -carotene, rubixanthin (10-15%) and traces of pro- γ -carotene were found in the two species of Chlorobium. The same carotenoids, but in larger amounts, were found in C. chlorochromatii (Mechsner [1957], Wittmann [1957]).

Goodwin showed that diphenylamine in concentration 1/40,000 suppressed the synthesis of γ -carotene and rubixanthin in Chlorobium, but there was an accumulation of small amounts (less than in purple bacteria) of phytoene, phytofluene, ζ -carotene and two colorless compounds of unascertained nature.

It should be noted that γ -carotene was first discovered in photosynthetic bacteria (Osnitskaya [1950], Goodwin [1956], Maksimova [1958]). This carotenoid, as Goodwin pointed out [1956, 1957], is more common in nonphotosynthesizing tissues and organisms — flower petals, fruits, fungi, and mycobacteria.

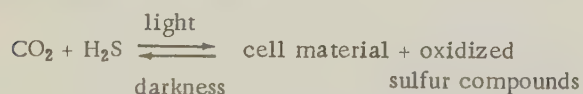
What role the carotenoids play in green sulfur bacteria is still obscure. It is remarkable, however, that they accompany chlorophyll in these organisms too. By analogy with plants and purple bacteria, we can postulate that the carotenoids or, more precisely, some of them, absorb light energy and transmit it to chlorophyll (Duyens [1952], Griffiths et al. [1955], Calvin [1955], Noggle [1957], Goodwin [1957, 1959], Goedheer [1959]).

Recently Stanier has expressed the hypothesis that the carotenoids preserve the cells from destruction due to oxidation sensitized by chlorophyll (Griffiths et al. [1955], Sistrom et al. [1956], Stanier and Cohen-Bazire [1957], Stanier [1958], Dworkin [1959]).

It has also been suggested that this is the function of carotenoids not only in photosynthetic, but also in heterotrophic nonphotosynthetic organisms.

Being photoautotrophs, green sulfur bacteria are associated in their development with the presence of light. No one has confirmed Nadson's claims [1912] that C. limicola is capable of growth in the dark. Obviously, these results indicate only that Chlorobium can remain for a long time in darkness without losing its viability.

From experiments with resting cells of C. thiosulfatophilum, Larsen [1953] claimed that these bacteria form certain acids, CO_2 , and H_2S , in darkness in anaerobic conditions. The last, in his opinion, proves the reversibility of the reaction:



We should also mention that green and purple bacteria often grow in water in fairly dark conditions at depth 13-14 m, to which only a small amount of radiant energy penetrates (Isachenko [1914], Kuznetsov [1942, 1952], Lyalikova [1957]). Photosynthetic bacteria fre-

quently develop under a dense layer of algae. In such cases their growth is possible only because the principal absorption maxima of the chlorophylls of plants and bacteria do not coincide (Stanier and Cohen-Bazire [1957]).

This does not mean, however, that the optimal conditions for the growth of green sulfur bacteria are created only at very low light intensity.

Larsen [1953] showed that the rate of CO_2 absorption by cell suspensions of C. thiosulfatophilum in the presence of H_2S or $\text{Na}_2\text{S}_2\text{O}_3$ increases considerably as the light intensity increases to approximately 100 erg/mm²·sec.

According to the data of Larsen, Yocum and van Niel [1952] and Larsen [1953], when a cell suspension of C. thiosulfatophilum is illuminated by monochromatic light with wavelength corresponding to the absorption maximum of chlorobium chlorophyll in the red region of the spectrum, the reduction of 1 M of CO_2 requires about 9 quanta of light (from 8 to 12), i.e., approximately as much as in the case of other photosynthetic organisms (van Niel [1954]).

The quantum yield was just as high when the bacteria were illuminated with blue rays, but was reduced in the presence of only green or yellow rays. Larsen [1953] believed that the changes in quantum yield in various regions of the spectrum were partially due to the fact that the green and yellow rays were absorbed by the Chlorobium carotenoid pigments, which are inactive for photosynthesis.

The quantum yield of C. thiosulfatophilum is not affected by the nature of the oxidizable compound (hydrogen donor) present in the medium.

The results of the conducted experiments were regarded as confirmation of the correctness of van Niel's view [1941, 1952, 1953, 1954] that the primary photochemical reaction in green plants and photosynthetic bacteria was of the same nature.

Green sulfur bacteria occur in various climatic conditions, from the northern seas (Nadson [1912]) to the salt lakes of Africa (Butlin [1953], Butlin and Postgate [1954]).

Kuznetsov [1942] observed large concentrations of Chlorobium in L. Bolshoe Kucheer at a depth of about 6 m, where the water temperature was 8-8.5°C. Kaplan [1956] noted the presence of green bacteria in sulfur springs with water temperature up to 30°C. At higher temperature, however, these microorganisms, in contrast to purple bacteria, did not develop. According to the data of laboratory investigations, the optimal conditions for the growth of different strains of Chlorobium are created at 25-30°C. Pelodyction, in contrast to Chlorobium, as Perfil'ev [1914b] indicates, normally develops at lower temperature (8-15°C); this organism withstands freezing, but a temperature rise to 27°C causes the rapid death of the cells.

It was noted by many authors, even in early works, that green sulfur bacteria can grow only in conditions of limited access of air. Nadson [1906, 1912] observed the

rapid death of Chlorobium in aerobic conditions and classed them as microaerophilic organisms.

Later, van Niel [1931] showed that green sulfur bacteria, like the purple bacteria, are strict anaerobes.

According to Baas-Becking and Wood [1955], green sulfur bacteria (Chlorobium) can grow only at Eh values -100 to -300 mv.

The optimal pH of the medium for the growth of all the known Chlorobium strains lies close to 7 (Kuznetsov [1952], van Niel [1931], Larsen [1952, 1953]). Their growth, however, is possible in the pH range from approximately 6-6.5 to 8.5-9 (van Neil [1931], Larsen [1952, 1953] or even 9.8 (Baas-Becking and Wood [1955])).

Enriched and pure Chlorobium cultures can be obtained relatively easily in the laboratory by the methods developed by van Niel [1931] and Larsen [1952, 1953].

A widely used medium for the culture of various species of Chlorobium is that of Larsen [1952, 1953], which contains (in %): NH_4Cl -0.1, KH_2PO_4 -0.1, MgCl_2 -0.05, NaCl -0.3, NaHCO_3 -0.2-0.5, CaCl_2 -0.01, $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ -0.1 (or $\text{Na}_2\text{S}_2\text{O}_3$ -0.1-0.2 or Na_2S -0.01). The medium is prepared from distilled water with the addition of (in $\mu\text{g}\%$): $\text{Fe}(\text{FeCl}_3\cdot 6\text{H}_2\text{O})$ -50, $\text{B}(\text{H}_3\text{BO}_3)$ -10, $\text{Zn}(\text{ZnSO}_4\cdot 7\text{H}_2\text{O})$ -10, $\text{Co}(\text{NO}_3)_2\cdot 6\text{H}_2\text{O}$ -5, $\text{Cu}(\text{CuSO}_4\cdot 5\text{H}_2\text{O})$ -0.5, and $\text{Mn}(\text{MnCl}_2\cdot 4\text{H}_2\text{O})$ -0.5.

The composition of this medium shows that Chlorobium is not fastidious as regards substrate. Their ability to grow on a defined medium of simple composition facilitates the task of discovering the role of individual elements in the life of the cells. However, the metabolism and importance of a large number of the elements contained in the culture media have not yet been studied.

We know that the requirement of certain compounds by green sulfur bacteria varies with the composition of the medium and the conditions under which the bacteria develop in nature. This applies, for instance, to NaCl . Chlorobium isolated from fresh water can grow in media without NaCl or in media with the addition of 0.1-3% NaCl (Mechsner [1957], Shaposhnikov et al. [1958]). However, for Chlorobium strains isolated from salt waters, where the salt content is sometimes very high (Isachenko [1927]), the presence of NaCl in the medium is essential. Chlorobium, like the purple bacteria, requires calcium for normal growth (Larsen [1953]). Mg is essential for the synthesis of chlorobium chlorophyll, and also for the photophosphorylation reactions accomplished by Chlorobium and purple bacteria (Frenkel [1956, 1959], Williams [1956a]). Co is evidently used by green bacteria for the synthesis of cobalamins (Kondrat'eva and Uspenskaya [1961]).

Of considerable interest are the data of Larsen [1952, 1953], who noted that when iron was deficient in the medium the growth of Chlorobium was weak and the cultures rapidly turned yellow. It was found that the quantity of chlorobium chlorophyll increases with increase in the Fe content of the medium up to 20 $\mu\text{g}\%$ per mg of cell nitrogen. Lascelles [1955] noted that when iron

was deficient the cells of green sulfur bacteria, like those of purple bacteria (Lascelles [1955], Voinovskaya and Krasnovskii [1955]), secreted porphyrins into the medium. Experiments with purple bacteria showed that the secretion of porphyrins was associated with the derangement of the synthesis of bacteriochlorophyll. Iron is involved in the formation of the precursors of bacteriochlorophyll. The pathways of synthesis of bacteriochlorophyll and chlorobium chlorophyll probably have common stages. Moreover, the iron requirement of Chlorobium is obviously associated with their content of cytochromes, the quantity of iron in which is about 0.37% (Kamen [1956]).

The presence of type C cytochromes in photosynthetic bacteria was discovered in a study of the facultative anaerobic purple bacterium Rhodospirillum rubrum (Vernon and Kamen [1953], Vernon [1953], Elsdon, Kamen and Vernon [1954]). Later they were found in strict anaerobes such as the purple and green sulfur bacteria (Kamen and Vernon [1954, 1955a,b], Newton and Kamen [1956], Kamen [1955, 1956], Bartisch and Kamen [1955, 1956, 1960]).

The cytochrome from C. limicola (called cytochrome 553) in the reduced state has absorption maxima at 553, 520, and 415 $\text{m}\mu$. It does not react with CO and is capable of slow autoxidation (Kamen and Vernon [1954], Kamen [1956]). The properties of the two cytochromes, 554 (I) and 554 (II), found in C. thiosulfatophilum (Gibson and Larsen [1955]) are very similar. A study of the spectral properties of reduced cytochrome 554 (I) showed that its absorption maxima occurred at 554, 523 and 417 $\text{m}\mu$. E'_0 of this compound at pH 7 is approximately 0.160 v. The cytochrome 554 (I) content of C. thiosulfatophilum is about 0.1% of their dry weight.

Because the cytochromes of purple bacteria are oxidized in the light and are reduced in the dark (Duysens [1954], Kamen and Vernon [1954], Kamen [1955, 1956], Chance and Smith [1955], Vernon [1959], Olson [1956, 1957, 1960]), it has been suggested that they are involved in the transfer of electrons from oxidizable compounds (hydrogen donors) to the hydroxyl-acceptor system, thus ensuring its reduction and continuous functioning during photosynthesis.

However, there are other hypotheses as to the role of the cytochromes in the photosynthetic process (Arnon [1959]).

In Baltscheffsky's opinion [1960] flavinadenine nucleotide is involved in a single chain of reactions with cytochromes in photosynthetic bacteria.

Peel [1958] indicates that C. thiosulfatophilum contains a considerable quantity of this compound, and flavin mononucleotide also.

The possibility of type C cytochromes participating in the transfer of electrons, not to oxygen, but to other compounds, is indicated by their presence in such anaerobic organisms as Desulfovibrio desulfuricans (Ishimoto et al. [1954], Postgate [1956]), Thiobacillus denitrificans (Aubert et al. [1958], Milhaud et al. [1958]).

The best source of nitrogen for green sulfur bacteria, just as for purple bacteria, is ammonium salts. It has also been shown that Chlorobium can use urea nitrogen (Shaposhnikov [1958]). Interesting results were obtained by Lindstrom [1950] and Fry [1955], who found that C. limicola, like many species of purple bacteria, can fix atmospheric nitrogen. From experiments involving N^{15} Wall, Wagenknecht et al. [1952] concluded that an intermediate product in the fixation of molecular nitrogen by photosynthetic bacteria, including green bacteria, was ammonia.

No less important an element for green sulfur bacteria, as also for other organisms, is phosphorus.

There is no doubt as to the very important role of phosphorus compounds in plant photosynthesis (Arnon [1956, 1959], Calvin [1956], Aronoff [1957], Noggle [1957], Losada et al. [1960]) and in various chemosynthetic organisms (Umbreit [1954], Belyaeva [1954], Sorokin [1954a,b, 1956], Milhaud et al. [1957]).

Experiments with intact cells of purple bacteria (Gest and Kamen [1948], Wassink [1949]), then with extracts of them, and finally with purified chromatophores (Frenkel [1954, 1956, 1958, 1959], Newton and Kamen [1956, 1957, 1958], Anderson [1958], Losada et al. [1950]) showed that these organisms, like green plants, effect the photophosphorylation of ADP to ATP with the concomitant absorption of orthophosphate from the medium. According to Arnon's theory (Arnon [1959], Losada, Trebst, Ogata and Arnon [1960]), the process of cyclic phosphorylation is the main link in plant and bacterial photosynthesis, since it is most closely connected with the conversion of light energy to chemical energy. In Arnon's opinion, the only chemical product which can be determined and which is formed as a result of direct absorption of light energy by photosynthetic bacteria is ATP. In plants the reduction of pyridine nucleotide in light also takes place.

As regards the green sulfur bacteria, Williams [1956] was the first to note that extracts of C. limicola cells in anaerobic conditions in light with AMP and Mg present brought about a decrease of orthophosphate in the medium and the formation, presumably, of ATP.

Working with intact cells of C. thiosulfatophilum, Shaposhnikov and Fedorov [1960] established that their illumination in the absence of CO_2 led to the absorption of orthophosphate from the medium and an increase in the quantity of polyphosphates in the cell, which were then used up in the dark fixation of CO_2 . The ATP level of the cells, however, remained constant all the time. Polyphosphates were also found in purple bacteria by Fedorov [1959].

It is obvious that polyphosphates in photosynthetic bacteria, as in many other lower organisms (Belozerskii and Kulaev [1957]), must be regarded as "energy accumulators," and ATP fulfils the role of an intermediary

in the transfer of labile phosphorus to other systems (Shaposhnikov and Fedorov [1960]).

It has been noted many times that green sulfur bacteria can grow at higher concentrations of hydrogen sulfide in the medium than some species of purple bacteria (Isachenko [1927], van Niel [1931]).

Yet Bicknell [1949] reported that she observed mass concentrations of these organisms in layers of water where the hydrogen sulfide content was low.

The results of laboratory experiments with various strains of Chlorobium show that the optimal concentration of $Na_2S \cdot 9H_2O$ in the medium for their growth is not more than 0.05-0.1% (Larsen [1953], Mechsner [1957], Shaposhnikov et al. [1958]). In the presence of 0.2% Na_2S the growth of green bacteria is greatly inhibited, and there is no growth at concentrations above 0.3%, whereas some of the purple bacteria still grow at such a hydrogen sulfide content.

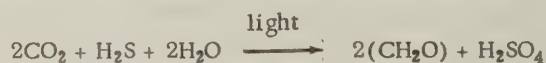
After van Niel's experiments with C. limicola [1931] it was believed for a long time that all the green sulfur bacteria could grow only in mineral medium containing hydrogen sulfide, which they oxidized to sulfur with the reduction of carbon dioxide. However, Larsen [1952, 1953] isolated a new strain of C. limicola which used up to 70% of the sulfur formed by the oxidation of hydrogen sulfide and converted this sulfur to sulfate.

The second species of green sulfur bacteria - C. thiosulfatophilum - which was first isolated by Larsen [1952, 1953], oxidized H_2S , S, $Na_2S_2O_3$, and $Na_2S_4O_6$. The ability to oxidize H_2S and $Na_2S_2O_3$ was also revealed by C. chlorochromatii (Mechsner [1957]).

Larsen's experiments with growing cells of C. thiosulfatophilum showed that these bacteria, like C. limicola, first oxidize hydrogen sulfide to sulfur, which is deposited in the medium. The process then goes further and all the sulfur is oxidized to sulfate:



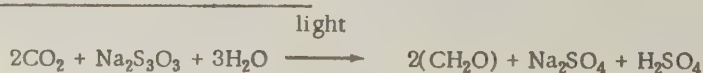
Hence, the final results of the oxidation of hydrogen sulfide by C. thiosulfatophilum, as in the case of the purple sulfur bacteria, can be expressed by the equation:



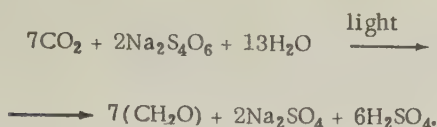
However, what the intermediate products of sulfur oxidation are have not yet been established. A test of the ability of C. thiosulfatophilum to use Na_2SO_3 gave negative results (Larsen [1953]).

The oxidation of thiosulfate by C. thiosulfatophilum has been studied a little more fully.

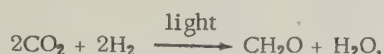
Larsen's experiments [1952, 1953] with cell suspensions of these bacteria showed that when they use thiosulfate for CO_2 reduction, as in the case of hydrogen sulfide oxidation, sulfates are formed in approximately the following relationship:



It is possible that one of the intermediate products of thiosulfate oxidation by C. thiosulfatophilum is tetrathionate, which these bacteria can easily use in the reduction of carbon dioxide (Larsen [1953]):



Trithionate, dithionate and sulfite are not oxidized by C. thiosulfatophilum, nor by C. limicola. Both Chlorobium species, however, like many of the purple bacteria, reduce CO_2 at the expense of molecular hydrogen. The results of this process are similar to the equation:



The greatest amount of carbon dioxide is fixed by C. thiosulfatophilum suspensions in an atmosphere containing 95-97.5% N_2 and 5-2.5% CO_2 .

Thus, in their ability to oxidize inorganic compounds all the species of Chlorobium, particularly C. thiosulfatophilum, reveal a great resemblance to the purple sulfur bacteria. This cannot be said of their behavior toward organic substances.

It is well known that sulfur and nonsulfur purple bacteria can bring about the photoreduction of carbon dioxide by oxidizing various organic compounds (van Niel [1941, 1944, 1952, 1954, 1956], Foster [1951], Gest [1951]).

It has also been established that some organic compounds, particularly acetic acid, can be used as a carbon source by purple bacteria (van Niel [1956], Elsdon [1955], Gest [1951], Foster [1951], Losada and Trebst [1960]).

As distinct from purple bacteria, C. limicola (van Niel [1931, 1941]) and C. thiosulfatophilum (Larsen [1953]) did not grow in media containing various organic compounds substituted for the hydrogen sulfide or hyposulfite (i.e., put in the place of the "hydrogen donor"). Larsen tried to culture C. thiosulfatophilum in media containing organic compounds of the RSH type (thiourea, thio-glycol, thioacetic acid, cysteine, etc.), carbohydrates or sodium salts of various organic acids. A very slight increase of biomass was noted only in media containing acetic, propionic or lactic acid. Beside this, Larsen found that the addition of many carbohydrates to a thio-sulfate-containing medium suppressed the growth of C. thiosulfatophilum, whereas organic acids had no such effect. It was noted, however, that the addition of pyruvic, succinic, malic, citric, or acetic acid to C. thio-sulfatophilum cell suspensions in anaerobic conditions caused the evolution of CO_2 . In the presence of lactic or propionic acid, particularly the latter, the amount of CO_2 in the medium decreased.

A study of this process showed that cells of C. thio-sulfatophilum in the presence of light, CO_2 , and propionic

acid formed succinic acid (Larsen [1951, 1953]). If C^{14}O_2 was present in the medium, the labeled carbon was almost entirely concentrated in the carbon of the carboxyl groups of succinic acid. This enabled Larsen to postulate that C. thiosulfatophilum was capable of effecting reactions of the type $\text{C}_3 + \text{C}_1 \rightarrow \text{C}_4$.

A study of the action of resting cells of C. thiosulfatophilum on pyruvic acid showed that the addition of pyruvic acid led to the evolution of CO_2 not only in the light but also in the dark. The degradation of pyruvic acid was more rapid in the presence of light. Two acid products which could not be identified were found in the medium, beside the CO_2 .

Thus, Larsen's investigations showed that C. thiosulfatophilum possesses enzymes capable of effecting some transformations of organic acids. However, normal growth of these bacteria could not be obtained in media containing organic compounds.

Hence, until recently it was believed that green sulfur bacteria were obligate photoautotrophs.

In 1957, however, there appeared Mechner's paper which indicated that C. chlorochromatii, as distinct from free-living Chlorobium species, could grow in pure culture in media containing such organic substances as peptone, malic acid, propionic acid or glycerol. A weak growth was also noted in media containing citric, butyric, acetic, or fumaric acid. Growth was observed only when light was present and in anaerobic culture conditions. In this case the addition of bicarbonate as a carbon source was not essential for the growth of the bacteria, except in the medium containing glycerol. The best substrate for C. chlorochromatii was a medium with 0.025% malic acid. A study of the utilization of this acid showed that most of it was converted to cell material and about 26-28% to CO_2 . Mechner believes that the ability of C. chlorochromatii to use organic compounds is due to the fact that in natural conditions they develop in symbiosis with a colorless bacterium.

Later, Sadler and Stanier [1960] isolated strains of C. limicola which used acetic acid in the presence of H_2S and CO_2 . A little before this there had been isolated free-living green sulfur bacteria which grew both in a mineral medium containing Na_2S and in media without Na_2S but with various organic compounds - alcohol, sugars, amino acids, or acetic or pyruvic acid (Shaposhnikov, Kondrat'eva et al. [1959, 1960]).

That these isolated organisms belonged to the green sulfur bacteria was indicated not only by the nature of their metabolism in mineral media, but also by the composition of their pigment system (Kondrat'eva and Moshentseva [1960]).

In morphology the isolated bacteria were similar to Chlorobium. They consisted of short rods, about 1-1.5 μ long. In contrast to Chlorobium, however, they possessed motility, due to the presence of a single flagellum (Shaposhnikov, Kondrat'eva and Fedorov [1959], Skalinskii

and Kondrat'eva [1961]). In view of this it was suggested that they should be assigned to the genus Chloropseudomonas (Czurda and Maresch [1937]).

The best development of the isolated bacteria was observed in a medium containing ethyl alcohol. The alcohol was still used in the presence of light, and its utilization was accompanied by absorption of carbon dioxide and the formation of acetic acid. An estimate of the carbon balance showed that the amount of carbon in the formed cells was always greater than the carbon dioxide carbon used up.

The pathway of carbon assimilation in green sulfur bacteria has not yet been studied. However, from information obtained in recent years in a study of this process in green bacteria, purple bacteria, and chemosynthetic organisms we can make some assumptions (Calvin [1955], Bassham and Calvin [1957], Glover [1952], Stoppani [1956], Trundinger [1956], Milhaud [1956], Aubert, Milhaud and Millet [1957, 1957a,b], Boichenko [1955], Doman [1959], Moses [1959], McFadden [1959], Losada et al. [1960], Losada, Trebst and Arnon [1960], Losada, Trebst, Ogata and Arnon [1960], Baugh et al. [1959]).

It is obvious, first of all, that fixation and reduction of carbon dioxide by photosynthetic organisms are not directly due to the presence of light. Larsen [1953] noted that C. thiosulfatophilum cells transferred to darkness absorbed a small amount of CO₂. The ability of C. thiosulfatophilum to fix CO₂ in the dark was confirmed by Shaposhnikov and Fedorov [1960].

It is possible that the first stages of carbon assimilation in various organisms, including green bacteria, may be similar under certain conditions. However, the later stages differ. Shaposhnikov [1957a, b, 1960] noted that one of the differences acquired during evolution between plant and bacterial photosynthesis was in the scale of this process. Photosynthesis in plants generally exceeds the requirement of constructive processes. Hence, there is a deposition of reserve substances such as starch, etc.

The functions of development and photosynthesis in plants can be isolated. In bacteria these processes are closely connected. Hence, the balance of bacterial photosynthesis is tilted toward the formation of amino acids and proteins.

In fact, it has been shown that assimilation of carbon dioxide carbon by Chromatium may proceed at first in the same way as in chloroplasts. The CO₂ is fixed in ribulose diphosphate and phosphoglyceric acid is formed. In purple bacteria, however, this acid is rapidly removed from the cycle and converted to phosphoenolpyruvic acid, from which oxaloacetic and aspartic acids are formed (Losada et al. [1960]). The use of acetate in constructive metabolism in these bacteria proceeds in another way. The first stage of photoassimilation of acetate is the formation of acetyl-coenzyme A, which is then condensed with various organic acids to form glutamic acid as the main product.

Thus, the experiments of Amon and his colleagues confirmed the wide abilities of purple bacteria in their ways of utilizing carbon compounds during photosynthesis. In view of the isolation of green sulfur bacteria capable of growing in organic media, we can infer that these organisms are capable of using for photosynthesis, not only carbon dioxide carbon, but also ready-made organic compounds.

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BIOLOGY OF LACTIC ACID BACTERIA

E. I. Kvasnikov

Uzbek SSR Academy of Sciences Press, Tashkent, 1960

Reviewed by V. M. Bogdanov

Translated from *Mikrobiologiya*, Vol. 30, No. 2,
pp. 364-367, March-April, 1961

The work being reviewed is devoted to a poorly studied group of lactic acid bacteria which are primarily associated with plants and with the conversion products of plant materials. The information available on these practically important microorganisms is very limited. E. I. Kvasnikov's monograph, which is the result of nearly twenty-five years of investigation, fills this gap.

In the introduction, the author emphasizes the tremendous importance of the lactic acid group of bacteria in human economy. He describes the ways that they are utilized in various branches of technology, and outlines the role of lactic acid bacteria as injurious agents in a number of food, and particularly in fermentation, industries.

The author gives a general characterization of the lactic acid group of bacteria. He shows the dependence of the shape of their cells on a number of factors, particularly on the composition of the medium. The question of the fermentative capabilities of lactic acid bacteria is illuminated. A definite relationship is established between the ability to ferment carbohydrates and the extent that the medium is supplied with amino acids and vitamins. The possibility is established of shifts from the heterofermentative type of fermentation to the homofermentative under the influence of certain factors.

The author made a thorough study of the alcohol resistance of lactic acid bacteria. He was the first to show that one of the characteristic properties of this group is its ability to grow in media with high concentrations of ethyl alcohol. At the same time, high acid-producing activity is always positively correlated with great alcohol resistance. The strains *Lactobacterium* (*Lactobacillus*) *buchneri*, *Lactobacterium* (*Lactobacillus*) *brevis*, and *Lactobacterium* (*Lactobacillus*) *fermentii*, isolated from defective wines, tolerate the greatest concentrations of ethyl alcohol. They grow in media containing 18-22° Balbing, and occasionally even 24 vol. % of alcohol. However, not only those strains that are isolated from wines, but also those from natural substrates (soils, rhizosphere, and epiphytic microflora of plants), are resistant to alcohol. Cocci are somewhat

less resistant than rods. The alcohol resistance of cultures decreases as they age. An interesting fact has been noted — the prolonged joint cultivation of lactic acid bacteria with yeasts leads to increased alcohol resistance. When lactic acid bacteria grow in media containing alcohol, the appearance of elongated cell forms is observed in a number of species. This phenomenon is explained by the fact that alcohol inhibits the function of division more markedly, whereas the ability to grow is inhibited to a lesser extent. Great resistance of lactic acid bacteria to aqueous solutions of ethyl alcohol is also described.

As is well known, the bactericidal effect of the individual representatives of the homologous series of monatomic alcohols increases as molecular weight becomes greater. It is shown that this rule is not applicable to lactic acid bacteria and that they, in distinction from all other microorganisms, exhibit the greatest resistance to ethyl, rather than to methyl, alcohol.

The high selective resistance of lactic acid bacteria to ethyl alcohol demonstrated by the author served as the basis for new selective media for the cultivation of these microorganisms. Prior to E. I. Kvasnikov's investigations, no such media had been proposed.

A truly selective medium for lactic acid bacteria consists of some base containing all of the required nutrient components, vitamins, and ethyl alcohol in a concentration that does not prevent the growth of these bacteria, but which has an inhibitory effect on the extraneous microflora. The proposed nutrient medium proved to be exceptionally convenient for the isolation of lactic acid bacteria from substrates containing a large number of different microorganisms.

During the investigation of these substrates, the isolation of lactic acid bacteria from them by commonly accepted methods was connected with a number of difficulties, because the growth of contaminating microorganisms suppressed the growth of colonies of the bacteria under investigation. On the basis of the proposed selective media, the author developed simple and convenient methods for the quantitative counting of lactic

acid bacteria, as well as methods of microbiological control.

In our work with lactic acid bacteria as employed in the dairy industry, we completely confirmed the correctness of E. I. Kvasnikov's conclusions. The use of alcohol-containing nutrient media is entirely expedient both for the enrichment of mixed cultures of lactic acid bacteria and for the isolation of pure cultures; this method is especially valuable in the investigation of the lactic acid microflora of plants and soil, which contain a relatively small number of the given bacteria. We only consider it necessary to emphasize that the concentration of alcohol added depends on the activity of the lactic acid microflora; the lactic acid bacteria of the southern and southeastern parts of USSR are distinguished by greater acid-producing energy as compared with regions of the central and northern parts (this pertains entirely to thermophilic lactic acid streptococci and rods); therefore, a somewhat smaller amount of alcohol (5-10%) should be added to the nutrient medium when investigating the lactic acid bacteria of the central and northern parts of USSR.

The utilization of new methods has enabled the author to carry out extensive investigations on the distribution of lactic acid bacteria in nature. He (together with M. G. Sumnevich) made a study of lactic acid bacteria in the epiphytic microflora of plants. This work showed that the content of lactic acid bacteria on plants varies within a wide range, and depends on a number of factors. It was shown that, on desert and semi-desert plants, either no lactic acid bacteria are found as a rule, or else only single cells per 1 g are detected. The author made the interesting observation that, in mountainous places which are completely inaccessible for the pasturing of livestock, lactic acid bacteria were found on higher plants, lichens, and mosses. There are many lactic acid bacteria on the surface of aquatic plants, particularly of algae.

Some important experiments are those which showed that strains of certain species of lactic acid bacteria (L. plantarum, Streptococcus lactis) are capable of migrating from the seeds which they were used to bacterize to the above-ground portion of the plants and to multiply there. On the basis of these experiments, it can be definitely stated that some lactic acid bacteria can multiply on the above-ground portions of plants.

E. I. Kvasnikov accorded much attention to the study of the soil and plant rhizospheres as habitats for lactic acid bacteria. The data which he obtained are essentially new and important. There was only occasional fragmentary information in the work of some authors concerning the detection of lactic acid bacteria in soil. We, simultaneously with E. I. Kvasnikov's investigations, also showed that the soil and plant rhizospheres are media from which lactic acid bacteria of some promise for the dairy industry can be isolated.

As the result of numerous investigations, the author established a number of distribution patterns for lactic acid bacteria in various soils, and a number of peculiarities of the nature of their interrelationships with plants in the rhizosphere were discovered.

The author showed that the number of lactic acid bacteria in the soil is largely determined by their properties. There are fewer of these bacteria in virgin soils than in cultivated ones.

The investigations of lactic acid bacteria should be continued from the geographical aspect.

The author makes the valuable comment that "the titer of lactic acid bacteria in soil is an indirect indicator of its richness in organic material and state of cultivation." It was established by means of direct laboratory experiments that lactic acid bacteria are capable of multiplying in soils and of being preserved in them for prolonged periods of time. As the result of numerous investigations, E. I. Kvasnikov came to the conclusion that lactic acid bacteria in soil become concentrated in the rhizospheres of both wild, and especially of cultivated, plants. Furthermore, there are more lactic acid bacteria in the rhizosphere of plants, as a rule, than in their epiphytic microflora. Thus, many lactic acid bacteria are typical rhizosphere microorganisms. Among the cocci found in soil and in the rhizosphere of plants are Streptococcus lactis, Streptococcus cremoris, Streptococcus durans, Streptococcus faecalis, and Leuconostoc mesenteroides, whereas among the rods, L. plantarum, and less frequently, L. brevis, L. fermentii, L. buchneri, L. leichmannii, and other species are found. The isolated strains varied greatly in activity. As a rule, their activity was lower than among epiphytic strains.

E. I. Kvasnikov's further investigations were directed toward the clarification of the peculiarities of the nature of the interrelationships of lactic acid bacteria with plants. As the result of a series of laboratory, greenhouse, and field experiments, it was found that, when seeds are "bacterized," the strains of certain species (L. plantarum and L. brevis) are capable of reproducing vigorously in plant rhizospheres.

The author's investigation of the role of lactic acid bacteria in the conversion of poorly-soluble phosphorus compounds in the soil are very interesting. The observations made demonstrated quite convincingly that lactic acid bacteria growing around roots assist in their assimilation of phosphorus from insoluble forms and, in this way, have a favorable influence on the growth of plants.

The experimental data showed that the natural habitat of the group of bacteria under investigation is the soil, where they become concentrated around the root systems of plants. Therefore, many lactic acid bacteria can be related to the typical rhizosphere microorganisms. When plant seeds are inoculated with lactic acid bacteria which are adapted to growth in their rhizosphere, they multiply

vigorously and are nourished to a considerable extent by root secretions. In a number of experiments, the author uncovered the peculiarities of the interrelationships between lactic acid bacteria and plants: they create a medium near the roots which is favorable for the growth of bacteria, whereas the latter favor the assimilation of phosphorus by the plant.

For the purpose of understanding the ecology of the microorganisms under investigation, the author gives a brief description of lactic acid bacteria inhabiting the human organism and animals. It is well known that Lactobacterium bifidum (Lactobacillus bifidus) is present in considerable numbers in the intestine of children and adults, but this microorganism has been poorly studied due to the lack of a well-developed method of counting.

The author correctly points out the diversity of the lactic acid microflora of the intestines; in addition to rod-shaped forms of lactic acid bacteria (L. acidophilus, L. fermentii, L. casei, L. brevis), lactic acid streptococci (S. faecalis, S. bovis, S. liquefaciens) are encountered in much greater numbers.

The species composition of the lactic acid bacteria inhabiting the animal organism and found on plants has a number of common characteristics and is specific. The lactic acid bacteria included in the composition of the epiphytic microflora play an important role in the formation of the microflora of the digestive tract.

The author's demonstration of a significant content of lactic acid streptococci in the intestine confirms our frequent recommendations concerning the necessity of introducing, in addition to acidophilic bacteria, lactic acid streptococci of intestinal origin (enterococci) into the microflora of dietetic dairy products. The necessity for this is due to the fact that the content of lactic acid streptococci is 100-1000 times greater than that of lactic acid bacilli; aside from this, lactic acid streptococci are constant inhabitants of the intestine; consequently, the effect of using dairy products which contain lactic acid streptococci and bacilli will be greater, because this will more rapidly restore the normal intestinal microflora in the sick organism.

One can not help but agree with the author that the intestinal tract is not an entirely favorable habitat for the acidophilus bacillus; in our observations, we frequently saw that, despite the regular consumption of acidophilus milk, the number of acidophilus bacilli in the intestine was not maintained at a high level and decreased quite rapidly after the omission of acidophilus milk from the diet.

The author's investigations devoted to the interrelationships of lactic acid bacteria and yeasts as a population having great significance in the food and fermentation industries are of great interest.

What is valuable in the monograph is the intimate mixture of profound theoretical investigations with the ability to pose and to solve a number of problems of national economic significance.

In our country, exceptionally great attention is accorded to the ensilage of feeds. Therefore, the working out of microbiological principles for the ensilage of feeds under various ecological-geographical conditions acquires special significance. As the result of the investigations conducted, new races of lactic acid bacteria for ensilage were suggested and a number of procedures for the biological preservation of feeds was recommended. The suggestions have found practical application.

The last section of the book is devoted to lactic acid bacteria that are agents of wine diseases. It is of particular interest and could have been the subject for a separate work.

E. I. Kvasnikov gives a complete critical review of the literature on the given question and shows that the considerable contradictions in the existing information are explained by the fact that many investigators lacked the correct approach in studying the bacteria that cause wine diseases. The microorganisms isolated from diseased wine were usually regarded as its specific microflora. Moreover, they were not studied in comparison with species isolated from natural substrates.

The author (together with G. F. Kondo) approached the study of the agents of wine diseases from the position of comparative ecology. This enabled him to discover a number of important biological principles concerning the nature of the adaptation of harmful microflora to industrial conditions. It was established that the diseases of wines are primarily caused by heterofermentative bacteria (chiefly by L. buchneri, L. brevis, and L. fermentii) and less frequently by homofermentative bacteria (L. plantarum). The processes of lactic acid souring of wines and biological acid reduction can not be linked only to narrowly specialized species, as is done by many investigators, and contrasted with species inhabiting the surrounding natural environment. The growth of lactic acid bacteria in wines is favored by their high adaptability to coexistence with yeasts.

The author shows a number of patterns in the course of fermentation by lactic acid bacteria, depending on the bacterial species and conditions of existence in wines.

Based on the knowledge of the biological properties of the agents of wine diseases, a system of procedures necessary to obtain healthy and stable wine was worked out. It is set forth in detail in the book being reviewed. Here, the author concentrates most of his attention on problems of prophylaxis. New methods of microbiological control are recommended for the most rapid detection of foci of infection. Much attention is devoted to questions of the selection of yeasts, and methods are proposed for the isolation of yeasts which raise the resistance of wine to diseases. The author's recommendations and the races isolated by him and his co-workers have found wide practical application and have contributed to the liquidation of wine diseases.

The following should be pointed out by way of general remarks.

A review should have been given of the basic literature devoted to the microbiology of milk and milk products, and a section should also have been included on the bacteriophage of lactic acid bacteria. This would have made the monograph more complete.

In the section dealing with the lactic acid bacteria of soil and of the rhizosphere of plants, the composition of the root secretions of plants which provide nutrients for lactic acid bacteria should have been reported.

In speaking of the nature of the effect of lactic acid bacteria on yeasts during their growth in a mixed population, the question should have been limited to the description of the peculiarities of action of individual species under specific conditions of the medium, without contrasting homofermentative bacteria with heterofermentative ones.

It would have been better if the contents of the monograph had been divided into separate chapters.

Correctly, the names of foreign authors should not have been written in Russian, since this unavoidably leads to errors.

In the text, when citing a literature source, it is simpler to indicate its ordinal number and to number the list of literature sources.

The figures in the book should have been given on a somewhat larger scale, which would have facilitated the reading of curves.

In conclusion, it should be especially emphasized that, as the result of E. I. Kvasnikov's work, we have a

new treatment of the ecology of lactic acid bacteria. Many aspects of the biology of these practically important microorganisms have become clear to us, particularly the characteristics of their interrelationships with the organic world. The established rule that, in natural and industrial substrates, lactic acid bacteria develop in close relationship with those organisms that, in comparison with them, have high auxoheterotropic properties, is very important. It is also quite true, as asserted by the author, that lactic acid bacteria satisfy their requirements for vitamins and amino acids by growing in the rhizosphere of plants (in root secretions and in the metabolic products of the auxoautotrophic microorganisms of the rhizosphere), in the intestinal tract of animals (apparently also principally due to the microflora inhabiting it), and in a number of sugar-containing substrates (chiefly among yeasts, in the company of which they multiply). Neither is there any doubt concerning the position that the high auxoautotrophic character of many lactic acid bacteria was shaped in the course of prolonged evolution with these representatives of the organic world.

E. I. Kvasnikov's monograph is a valuable contribution to science and is important in practice.

The Academy of Sciences of the Uzbek SSR Press did a big and good piece of work in publishing this book which is so necessary to wide circles of scientific and industrial workers. However, the circulation of the book—one thousand copies—is clearly inadequate.

SEXUALITY IN BACTERIA

E. L. Wollman and F. Jacob

Paris, Masson 1959; 247 pages,
illustrations; price 3000 francs

Reviewed by A. S. Kriviskii

Translated from *Mikrobiologiya*, Vol. 30, No. 2,
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In 1959 Wollman and Jacob published a specialized monograph dealing with the problem of sex in bacteria. Beside a general survey of the problem of sex in bacteria and some sections on bacterial genetics, the book also presents the authors' original work on two mutually connected questions — the conjugation mechanism in bacteria, and their inheritance of lysogenesis.

The monograph is divided into 14 chapters, comprising four sections.

The first section (Chap. I-II) briefly describes basic research in the field of bacterial genetics. This presents data from the literature on bacterial variation, contemporary methods for studying spontaneous and induced bacterial mutations; it also discusses the currently known means by which bacteria transfer their hereditary traits — i.e., recombinations, transformations, transduction, lysogenation (lysogenic conversion) — with a description of the nature of lysogenesis and its genetic significance.

The second section (Chap. III-IV), likewise a survey, is devoted to works on bacterial recombination belonging to an early period of the study of this problem — mainly, it presents Lederberg and Tatum's work with *Escherichia coli* strains K-12, which yield recombinations with a low frequency. Here we have a detailed description of genetic analysis methods of the results of cross-breeding; a listing of the characteristics most frequently applicable in conducting such experiments; a discussion of the meaning of allelism and of the heterozygous state in genetic recombinations; a description of sexual differentiation in bacteria (the fertility factor F). The authors indicate that there currently exist two hypotheses attempting to explain the bacterial genetic recombination mechanism; that of Lederberg and Cavalli, which maintains that bacterial conjugation is a process differing but little, basically, from the sexual process of other organisms, and that it leads to the formation of a zygote in which the genes of both parental types are equally represented; then there is the hypothesis of Heiss, who considers that bacterial conjugation differs basically from conjugation in other organisms, and that it produces an incomplete zygote, which includes only some parts of the donor

bacteria's hereditary apparatus. Heiss' point of view receives support from the research of the authors themselves, which is presented in the third section of the monograph (Chap. V-IX). This important section of the book, devoted to a study of high frequency bacterial genetic recombinations, describes thoroughly the results obtained when crossing the so-called Hfr strains (Hfr — high recombination frequency) with F-strains. In accordance with Heiss' data, in this type of cross-breeding there occurs, upon conjugation, a transfer of some properties of the Hfr donor strain to the F-receiver strain. The section contains four chapters. The first of these (Chap. V) gives a general characterization of cross-breeding of this type. It presents the discovery histories and the characteristics of the various Hfr strains; crossing methods and genetic analyses of recombinants developed by the authors; as well as experiments on the transference of certain traits from the prototrophic Hfr strains to the auxotrophic F-strains. In these experiments the authors managed to establish that the various traits are transferred with varying frequencies, and that the number of arising recombinants depends on the duration of contact of the parental strains and also on the nature of these strains. On the basis of genetic cross-breeding experiments, Chapter VI discusses a subject of great importance and interest not only to microbiologists and geneticists, but also to virologists — the question of prophage localization in the genetic apparatus of lysogenic bacteria. After a number of painstaking and clever experiments, the authors decided that the factor determining the lysogenic state of the bacterial cell, i.e., the prophage, behaves in a way similar to any other hereditary cell factor. From this the authors conclude that in lysogenation the genetic material of the infecting phage, which consists of deoxyribonucleic acid threads, is fixed onto the bacterial "chromosome" and is reduplicated synchronously with the genetic material of the bacterial host cell. In the authors' opinion, the phage gene enters the bacterial cell gene, not by replacing a homologous bacterial "chromosome" part, but rather by joining one of its specific components. Herein lies the real difference between the prophage and other hereditary

factors of the bacterial cell. Chapter VII deals with the so-called zygotic induction discovered by the authors. This interesting phenomenon (comparable to ultraviolet ray induction of lysogenic cultures) consists of the fact that upon crossing the lysogenic Hfr strain with the non-lysogenic F⁻, the prophage, entering the resulting zygote, develops into a mature phage, and the zygote perishes by phagolysis. The authors describe methods for obtaining zygote induction, its mechanism, and the relation between zygotic induction and location of the prophage on the bacterial chromosome. Since the extent of zygote induction is easily determined by the number of developing sterile spots, this method affords the microbiologist-geneticist a very accurate and easy means to measure the transference frequency of a given trait (in this case of lysogenic ability, consequent on the presence of phage) from the donor bacteria of lysogenic Hfr strain to the receiver bacteria F⁻. Specifically, the authors used this method to study the kinetics of conjugation, and the frequency of zygote induction showed that conjugation occurs frequently—in almost half of the lysogenic strain Hfr cells. If this is so, one should be able to observe conjugation directly with a microscope. And indeed, using a highly original marker for one of the parental forms by adsorbing the phage λ onto the cells of the receiver strain (the receiver strain was a phage-sensitive; the donor strain, a phage-stable, culture of *E. coli* strain K-12), the authors obtained highly illustrative electron photomicrographs, which clearly show that conjugation takes place between genetically differing cell types. These interesting investigations are described in Chapter VIII of the monograph. The last chapter of the book's experimental part, Chapter IX, discusses the transfer mechanism of genetic material in the course of the conjugation process. The authors showed that the hereditary traits are always transferred in one and the same definite order—they demonstrated this fact by violent shaking of the Hfr-F⁻ bacterial mixtures at various time intervals after mixing (thus artificially separating the conjugating pairs), and studying the nature of the traits transferred to the receiver strain.

Based on this they present the following hypothesis for the mechanism of genetic transfer in conjugation: after the formation of a connecting cytoplasmic bridge, the chromosome of the donor bacterium penetrates the receiver bacterium with one of its ends (it is always the same end), and gradually enters the receiver cell completely. During this period, which lasts several tens of minutes, spontaneous ruptures of the progressing chromosome are possible, and this causes an incomplete transfer of traits. The chance for the transmittance to the zygote of any specific trait depends on the location of the corresponding hereditary factor upon the donor bacterium's chromosome: the factors most distant from the penetrating end stand little chance of transmission; those closest located will practically always appear in the zygote.

The last section of the book (Chap. X-XIV) is of a general nature. Here the authors, on the basis of their own and the literature data, express theoretical thoughts on questions of recombinations of bacteria possessing differing characteristics; they attempt to systematize the various bacterial sexual types; they discuss the role of the prophage and other latent viruses in the variation of hereditary characteristics of the host cell.

Of considerable interest are the authors' hypotheses on the differences between the F⁺ strains, characterized by a low recombination frequency, and the Hfr strains. Thus, in the F⁺ strains the chromosome has a ring-like structure; the sex factor (F) is not connected to the chromosome. Therefore, upon crossing F⁺ \times F⁻, the sex factor is transferred with a high frequency (in a mixed culture of F⁺ and F⁻, the F⁻ cells are very easily transformed into F⁺), whereas the remaining traits, determined by factors located in the locked chromosome ring, do not, as a rule, pass into the zygote. The strain Hfr is a mutant of strain F⁺. The mutation consists of the rupture of the chromosome ring in some section, and of the union to its distal end of sex factor F. As a result of this mutation the chromosome becomes capable, during conjugation, of penetrating the receiver bacterium F⁻ with its proximal end; the F⁻ is lacking in the sex factor and possesses a locked ring-form chromosome. As to the nature of the sex factor F, which determines to which sexual type the bacterium belongs—to the "male" (donor) or to the "female" (receiver strain)—it is as yet unclear. It is only known that in the F⁺ strain the sex factor is not joined to other characteristics of the cell, is capable of autonomous multiplication, and apparently exists in the donor cell in several numbers, one of which passes into the receiver strain F⁻ during conjugation.

Considering the ability of prophage to completely incorporate itself into the genetic apparatus of the host cell, the authors introduce a new term: "episome." By episomes they designate genetic elements capable of existing both in an integrated state in relation to the host cell, as well as autonomously. In the authors' opinion, there exist within the bacterial cell three types of elements with episomatic qualities—tamed phages, bacteriocins, and the sex factor F. These elements comprise a class of structures intermediate between the normal hereditary factors of the bacterial cell, and the typical infective viruses, which are external in relation to the cell.

These are the basic contents of this book, which to this day is the only one in the world literature devoted to questions of sex in bacteria. The book ends with a general conclusion and a carefully compiled bibliography encompassing some 400 titles. Several tens of schematic diagrams and graphs aid in mastering the material, which is laid forth in simple, clear language. In spite of its comparatively short length, the book is saturated with a huge number of experimental facts which leave no doubt that the transfer of hereditary traits in bacteria by

means of recombinations, at the basis of which lies a physical union (conjugation) of two genetically different cells, is a firmly established fact. It is regrettable, of course, that in spite of the importance of bacteria to general genetics and biology, the experiments thus far have been conducted on a relatively insignificant number of subjects — mainly confined to the intestinal group of bacteria. Now, after the basic fact has been established, the next problem of microbiologists is to extend these investigations to include the various representatives of the greatly diversified bacterial world.

Although the factual side of the experimental material presented in the book does not arouse any doubts, it is difficult to agree placidly with all of the authors' theoretical views, although some of these doubtlessly deserve serious attention as well-founded working hypotheses. Especially hypothetical is the authors' assumed existence of differences between the Hfr and the F+ strains and the ring structure of bacterial chromosomes. It would be very important to confirm these structures by morphological data, which is quite possible with the present day development of electron microscope technology.

It is definitely incorrect, from our point of view, to include tame phages in the episome concept. There can be no doubt that tame and virulent phages belong to one and the same group of living systems and are even closely related genetically. There can also be no doubt that the virulent phages are typical bacterial viruses, cell parasites. They are, by their nature, at least at the present stage of evolution, exogenous in relation to the cell. The tame viruses are the same type of cell parasites, although they did acquire in the process of evolution the ability to incorporate completely, temporarily, into the cell's genetic apparatus and to play the role of genetic factors during this period. This parasitism and symbiosis on the genetic or molecular level is, apparently, one of the cardinal characteristics of viruses as absolute intracellular parasites, but it tells nothing of their origin (whether endogenous or exogenous). All the basic traits of viruses and phages — their ability for autonomous adaptive variation and evolution, their surprising and varied mechanisms for adaptation to survival in the external medium and for attacking the host cell, their wide spread among the attacked hosts, and many other traits — characterize them as independently existing living systems, very well adapted to intracellular parasitism, but not as parts of the cell structures.

By "episomes" the authors of the reviewed book designate, primarily, hereditary elements of the host cell which are capable also of autonomous development. The inclusion in this hypothetical class of tame phages — typical representatives of viruses — deprives these of their basic characteristic as exogenous parasites. Such a point of view, aside from all other considerations, disorganizes the practice of developing rational fighting measures

against these parasitic pre-cellular organisms in the various fields of national economy and medicine. We must note that a view on the nature of viruses, which is close to that of the above authors, but in our opinion even more incorrect, has been expressed in very recent years by some foreign scientists, especially by the famous American virologist and bacteriophage specialist, S. E. Luria. In a number of his recently published theoretical articles and surveys, Luria identifies viruses as "elements of genetic material, which enables their introduction into other cells." * In other words, viruses are wandering genes which have acquired, aside from their basic qualities, the ability to don fur coats, thus exit into the external environment and then penetrate into other cells, where they are again transformed into typical genes. There is no need to emphasize that this identification does not include the basic qualities of viruses — their ability to disorganize cellular metabolism, directing it to their own need, converting the normal cell into a pathological one and frequently killing it. Since a detailed analysis of the above-mentioned views would require a separate article, we can only note here that the number of similarities discovered in recent years between viruses and endogenous cellular hereditary factors gives no basis for equating these two completely different (from the biological viewpoint) categories, whose only similarity is that, having the same material basis (nucleic acids or nucleo-proteids), both (viruses and genes) display their action on the molecular level.

Evaluating the book as a whole, we wish to emphasize that, aside from the problem of sexual differentiation in bacteria, the material presented concerns a number of other very timely questions for biology and medicine — specifically, the question of latent virus infection, and thus it is of great interest to Soviet biologists and medical men. The main subject of the monograph — sex in bacteria — is presented with extreme thoroughness. Since work on bacterial sexuality is not as yet being conducted in the Soviet Union, and considering the practical significance of such work for obtaining new, valuable kinds of microbes, the familiarization of Soviet microbiologists with the monograph would be highly desirable, and would doubtless stimulate scientific research in this field. The book is also of interest to virologists working on problems of latent virus infection, to researchers in experimental oncology, to workers with bacteriophages, and to geneticists in various fields. In spite of some disputable theoretical assumptions, the book, as the only survey-experimental monograph of its kind on sexual differentiation in bacteria, is surely worth translating into Russian.

* Luria, S. E. "Viruses as infective genetic materials." In: *Immunity and Virus Infection*. Ed.: V. A. Najjar (John Wiley, New York, 1959) p. 188.

FIRST INTERNATIONAL SYMPOSIUM IN ITALY

I. V. Konova, M. V. Fateeva,
and N. D. Ierusalimskii

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The First International Symposium on Fermentation was held in Rome on May 9-14, 1960. The symposium was organized by the Istituto Superiore di Sanita, the Societa Chimica Italiana, and the Fermentation Subdivision of the American Chemical Society. The sessions of the symposium were held in the Istituto Superiore di Sanita and 300 representatives from 24 countries took part. The largest delegations were those from Italy (101), England (44), USA (27), and the USSR (19).

In addition to specialists working in the particular fields of microbiology, representatives of certain industrial firms took part in the symposium.

About 30 papers altogether were heard, five of them being read by members of the Soviet delegation. All the papers were translated into three languages — Italian, English, and Russian. The symposium was opened with an address by the Director of the Istituto Superiore di Sanita, D. Marotta, who briefly described the work of this institute and of the Societa Chimica Italiana. He was followed by: E. B. Chain, the Nobel Prize Winner, who is head of the Biochemistry Division and Director of the International Center of Chemical Microbiology in the Istituto Superiore di Sanita in Rome; J. C. Sylvester, President of the Fermentation Subdivision of the American Chemical Society; and, finally, the Italian Minister of Health.

In their speeches they noted the importance of the International Symposium on Fermentation and welcomed the participants.

The papers presented at the symposium dealt with three problems:

1. Technology of fermentation processes.
2. Research on individual fermentation processes.
3. Genetics of microorganisms producing antibiotics and other specific substances.

In the discussion of the technology of microbiological processes considerable attention was given to questions of aeration in submerged fermentations.

The paper of E. L. Gaden (USA) dealt with the optimal conditions of air supply and mixing of the culture fluid. He gave examples of calculations of the oxygen requirement in fermentations with reference to its degree of solution, adsorption, etc.

Italian scientists A. Carilli, E. B. Chain, G. Gulandi and G. Morisi presented a very interesting paper giving data on the oxygen conditions in processes for obtaining yeast, tetracycline and penicillin. These authors believe that a mechanical method of mixing is better, since it is more economical than mixing solely by a stream of air. These authors noted that the morphology of the producer mycelium was of great importance for producing a particular degree of viscosity in the culture medium. Numerous experiments with various fungi in fermentors of different shapes and sizes had shown that with an air flow of 0.3 to 1 volume/min and a particular degree of mechanical mixing the culture fluid in every case contained excess oxygen, provided that the dry weight did not exceed 2%, and the viscosity was not more than 6 units. Special apparatus designed in the Istituto Superiore di Sanita was used for continuous measurement of the dissolved oxygen in fermentors of different capacities (these fermentors were described in a paper by R. Falini).

In the paper "Oxygen transfer in fermentations," M. J. Johnson and D. H. Phillips (USA) reported that when aerobic bacteria and molds were cultivated in 3-liter and 1900-liter fermentors an oxygen deficiency was observed at a dissolved oxygen pressure of 0.01-0.02 atm for bacteria, and 0.07-0.15 atm for molds. The oxygen transfer factor in the case of bacteria varied with the dimensions of the fermentor, being higher in the small fermentors and lower in the large fermentors, whereas in the case of fungi the factor was very low in both cases.

The effect of aeration under pressure on microorganisms was dealt with by C. G. Héden and A. S. Malmberg (Switzerland), who gave data on the suppression of growth of *Escherichia coli* and *Staphylococcus aureus* under pressures above 5 kg/cm². These authors attributed the toxic effect of oxygen under pressure to the fact that oxygen is a double radical and is capable of reacting with "unstable hydrogen" contained in some constituents of the medium (cysteine, glutathione), thus leading to the production of peroxides.

The paper by R. K. Finn (USA) gave some information on the harmful effect of friction on microorganisms in the case of strong mixing. The work had been per-

formed with molds and actinomycetes in experiments in which a special apparatus was used.

An account of measures for the prevention of foam formation in industrial conditions was given by Canadian scientist S. S. Dawson.

T. Wiken (Holland) gave some information on the effect of aeration on fermentation of sugars in distilled water and buffer solutions by the yeasts Saccharomyces vini and Bretanomyces. This author believes that the absence of the Pasteur effect is a characteristic feature of Bretanomyces and can be used for their identification, although at the same time he noted the absence of the Pasteur effect in S. vini in certain conditions (in buffer solutions).

An account of the adaptive regulation of the pH and rH_2 of the medium was given by I. L. Rabotnova (USSR), who cited examples of alteration of the pH and Eh of the medium by various microorganisms in accordance with their requirements.

The topical problem of designing a reliable and convenient system of pH measurement in fermentation processes was dealt with by J. Di Spigno (USA), who gave a detailed description and diagrams of instruments for the continuous measurement of pH in fermentors. The employed electrodes ensured trouble-free operation for 1.5 years, and could withstand continuous or periodic sterilization by autoclaving.

Several papers were devoted to questions of continuous cultivation.

The paper by Corresponding Member AN SSSR N.D. Ierusalimskii gave a classification of methods of continuous cultivation and discussed the employment of particular methods in the tackling of practical problems.

I. Málik (Czechoslovakia) gave a more accurate treatment of the theory and practice of two stage continuous fermentations.

J. S. Pirt and D. S. Callow (England) described an industrial method for the continuous production of penicillin in a two-stage system.

The papers dealing with the study of individual fermentations discussed problems of the biosynthesis of antibiotics, vitamin B₁₂, and ergoalkaloids, steroid transformations and the oxidative activity of microorganisms.

G. N. Rolinson (England) dealt with the production of 6-aminopenicillanic acid, the core of penicillin, which can then be subjected to purely chemical treatment to obtain various derivatives of this antibiotic, including those which act on microorganisms resistant to natural penicillin. Rolinson reported that the biosynthesis of this acid was stimulated by aeration up to a certain point, above which there was observed a suppression of the biosynthesis of 6-aminopenicillanic acid, while the biosynthesis of penicillin was still stimulated. Data on the chemical and antibiotic properties of 6-APA were given in the paper.

W. M. Stark (USA) reported a new antibiotic, tylosin. Tylosin (C₄₅H₇₇NO₁₇, molecular weight 904), with

an antibacterial spectrum which includes gram-negative and gram-positive organisms, shows great promise for veterinary medicine and animal husbandry. A study of the formation of tylosin showed that this process took place in two stages, the first stage being characterized by rapid growth of the mycelium, and the second by production of the antibiotic. The author suggested a synthetic medium for biosynthesis of the antibiotic. He has made a study of the individual components, including methylolate, the addition of which considerably increases the yield of tylosin.

A study of the conditions of biosynthesis of oxytetracycline by Actinomyces rimosus was dealt with by N. V. Orlova (USSR), who showed that the development of A. rimosus proceeded in two phases. The first phase — the growth phase — requires the presence of certain amounts of nitrogen, carbohydrates, and inorganic phosphorus in the medium; the second phase proceeds in the complete absence of inorganic phosphorus, but the presence of carbohydrates is essential. The optimal conditions for biosynthesis of the antibiotic are provided when the constituents of the medium are present in certain proportions.

The paper of H. Umezawa (Japan) was devoted to anticancer antibiotics.

Di Marco in Italy is occupied with problems of vitamin B₁₂ synthesis. His paper (A. Di Marco, G. Boretti, L. Spalla, A. Migliacci) at the symposium dealt with the biosynthesis of cobalamins by Nocardia rugosa. The author claimed that the laboratory-produced "Farmitalia" mutants of N. rugosa are distinguished by their ability to produce B₁₂ and porphyrins. In a study of the pathways of B₁₂ and porphyrins, Di Marco found that the addition of δ -aminolevulinic acid to a N. rugosa culture led only to a slight increase in porphobilinogen and porphyrins, but had no appreciable effect on B₁₂ synthesis. Of great interest were Di Marco's investigations on two N. rugosa mutants incapable of synthesizing B₁₂ or other growth factors for E. coli. When cultured together, these strains produced up to 2-4 μ g of B₁₂ per ml, and this led the author to conclude that there is a microbiologically inactive vitamin B₁₂ precursor, which probably contains cobalt. The author found that the guanosine diphosphate of factor B and the phosphate of factor B were intermediate products in the reactions leading to the construction of the complete B₁₂ molecule. An account of observations on the growth of a submerged Claviceps culture was given in a paper by A. Tonolo, T. Scotti and L. Vero (Italy).

Steroid transformations were the subject of papers by C. E. Holmlund (USA) — "Substrate specificity in the microbiological transformation of steroids," and R. W. Thoma (USA) — "Dehydrogenases of the steroid ring in microbial cells and cell-free systems."

The microbiological oxidation of hydrocarbons and their derivatives (methane, ethane, heptane, naphthalene, etc.) was reported on by C. Arnaudi and V. Treccani

(Italy). C. Antoniani's (Italy) paper was also concerned with the oxidative activity of bacteria.

There were several papers on the genetics of microbial producers of antibiotics and other specific substances.

The papers of J. A. Roper (England) and G. Sermonti (Italy) were devoted to the study of the parasexual cycle in molds. The term parasexual cycle was introduced in 1954 by Pontecorvo to designate the exchange of nuclei in hyphal anastomoses where, as distinct from the sexual process, the nuclei do not fuse together.

The parasexual cycle enables the crossing of fungal strains with different characters and with no sexual cycle.

In Sermonti's paper on the use of the parasexual cycle in the penicillin industry, he discussed the possibility of obtaining mutants synthesizing a modified antibiotic molecule.

J. F. Stauffer (USA) reported obtaining highly productive strains of *Penicillium chrysogenum* by exposure to ultraviolet; the penicillin yield was increased from 100 units/ml to several thousands of units/ml. This author has isolated in the laboratory a pigmentless *Penicillium* strain, which is being used as an initial strain for further work. Research has recently been started on cephalosporin producers, particularly *Emericlesopsis terricola* var. *glabra*.

The paper by D. A. Hopwood (England) on the life cycle of *Streptomyces coelicolor* gave an account of morphocytological observations on this organism.

The problem of microbial selection was the subject of a paper by V. I. Kudryavtsev (USSR), who proposed a method of continuous improving selection of industrial yeasts.

The problem of the appearance of antibiotic-resistant forms of bacteria was discussed by Littoria (Italy).

The paper of K. Kitahara (Japan) contained a description of elongated forms of lactic acid bacteria appearing in unfavorable conditions.

Beside participating in the work of the symposium, the delegates were given the opportunity to visit some research institutions in Italy. Through the kindness of Professor Chain the Soviet delegation was able to acquaint itself with the trend of research in the Istituto Superiore di Sanita, which is the largest medical research center in Italy. This Institute, which occupies several buildings (the area of the main building is 53,000 m²) has a staff of 800, of which 200 are graduates. The Institute produces sera, vaccines, and penicillin.

The Biochemistry Division, directed by Professor Chain, is studying the intermediate stages of carbohy-

drate metabolism in the human organism. This Division includes the laboratory called the Center of Chemical Microbiology, where research is being conducted on obtaining 6-aminopenicillanic acid, the biosynthesis of citric acid by means of submerged fungal cultures, and the development of a method for obtaining ergoalkaloids (derivatives of lysergic acid) by means of pure cultures of the ergot *Claviceps*. These alkaloids are used in the treatment of cardiac and gynecological diseases.

There is a pilot plant in the Center of Chemical Microbiology. The staff of the Institute includes qualified technicians, who are engaged in the design of original apparatus. We were impressed by the ingeniously designed system of instruments for the quantitative determination of the components of a mixture of substances labeled with radioisotopes and separated by two-dimensional chromatography. With the aid of this apparatus it is possible to make a comparative study of the metabolic products of normal and pathological tissues, which are characterized by particular proportions of certain metabolites (amino acids, organic acids, glycogen, etc.). This allows a rapid differentiation of pathological tissues, e.g., tumor tissues.

The Soviet delegation visited Milan University, where they became acquainted with the work of four departments.

In the Department of Microbiology, headed by C. Arnaudi, research is being conducted on diverse questions of general industrial and soil microbiology. Particular attention is being given to the transformation of steroids and the oxidation of carbohydrates by microorganisms. The plan of work of the Department of Biochemistry, headed by Professor Antoniani, includes, in addition to biochemical studies, several topics relating to the oxidative activity of acetic acid and other bacteria.

The Department of Phytopathology, headed by E. Baldacci, is devoting considerable attention to research on the use of antibiotics for the control of crop diseases.

In the Department of Plant Physiology, headed by Professor Marre, unicellular algae are being studied in addition to other topics.

In conclusion we must mention that, beside the efficient organization of the work of the symposium, the participants at this congress in Italy encountered great interest in their work on the part of the President of the Italian Republic. This was manifested in the reception which he held for the delegates to the symposium.

MICROBIOLOGICAL RESEARCH IN HUNGARY

S. V. Goryunova and L. K. Osnitskaya

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During our mission to the Hungarian People's Republic we visited several scientific institutions and acquainted ourselves with the work being conducted there. Our first visit was to the Microbiological Institute (or Department, as we would call it) (Director: J. Banhegyi) in Budapest University.

For several years this Institute has been engaged in a thorough study of the taxonomy of the lower fungi with the aim of compiling a key, the first volume of which is now ready for the press and will be published simultaneously in Hungarian and German. In addition to this work, Professor Banhegyi and his colleagues are occupied with research on petroleum microbiology.

Investigations are being conducted along two lines: 1) to find out if it is possible to apply the method of bacterial prospecting for oil and gas by means of bacteria oxidizing methane, ethane or propane and 2) to put bacteria to practical use for releasing petroleum from exhausted wells.

As regards the first line of research several hundreds of species of bacteria have been isolated from the soils of oil-bearing deposits in Hungary. Twenty of these species developed only on organic media in an atmosphere of methane, ethane or propane. The most active was Nocardia coralina, which develops in an atmosphere of propane.

Tests of the obtained cultures in field conditions showed that their activity varied with the season in which the experiments were conducted. The activity of the cultures was also affected by the low temperature below the soil surface (to avoid the effects of surface processes the cultures were buried at depth 1.5 to 3 m during the experiments). Hence, the investigators had to conduct special experiments to train the cultures to low temperatures. Success in training the cultures to low temperatures was achieved by the addition of p-aminobenzoic acid and hemin (0.5 μ g each) to the medium.

On the second topic — the practical employment of bacteria for releasing petroleum — Banhegyi's colleagues have conducted preliminary experiments with mixed cultures which were used to enrich media containing petroleum and molasses. The experiments revealed a considerable lowering of the viscosity of the petroleum. In the laboratory Banhegyi kindly demonstrated to us the specially designed apparatus in which these experiments

were conducted. The experimental procedure is essentially as follows: Fermentation of the molasses leads to the formation of surface-active substances (gaseous and liquid products) which, in passing through a layer of sand containing petroleum, reduce the force of cohesion between the sand particles and the petroleum, thus reducing the viscosity of the latter and assisting its separation from the sand. Only mixed cultures of thermophilic bacteria with an optimum of development at 50°C (clostridia, sulfate-reducers, nitrifiers, etc.) were used, as these were most active. Industrial experiments involving the pumping of a nutrient solution containing molasses and bacteria into exhausted wells are planned for the summer of 1960.

Beside the above work, the staff of the Institute is occupied with studies of fungal diseases of insects and other questions.

Considerable research on fungi is also being conducted in the Ministry of Agriculture Museum of Natural Science in Budapest, where pure cultures both of lower and of higher fungi are maintained.

The director of the Institute of Plant Physiology in Budapest Humanitarian University is V. Frenyo. This department is mainly occupied with an elucidation of the laws involved in the consumption and utilization of salts by different crops in different periods of development of the plants. For this purpose Professor Frenyo has devised rapid methods of determining certain mineral salts (phosphates, nitrates, etc.) in solutions. These methods could be used effectively in practical microbiological research.

Docent Feher is engaged in research on sulfate reduction in higher plants. In view of the fact that sulfur compounds (cystine, cysteine, methionine, etc.) in plant cells are labile, Feher has given special attention to methods of determining these substances. He has chosen yeasts as indicator organisms for detecting the presence of various sulfur compounds in solutions, since it is well known that certain genera of yeasts use only organic sulfur, while others reduce sulfates. In Feher's opinion, the laws governing these processes are different in the lower fungi. Fungi will also be used for these investigations.

The director of the Institute of Plant Physiology in the University of Szeged is I. Szalai. In addition to the

lectures and studies directly connected with plant physiology there is a special course on general microbiology in the Institute and some microbiological research is being carried out. For instance, L. Ferenczy is engaged in research on bactericidal substances secreted by higher plants. In particular, he has isolated an antibiotic which is active against mycobacteria.

This antibiotic has now been obtained in crystalline form (with the cooperation of the staff of the Institute of Organic Chemistry). Tests on animals have been made and it has now been accepted for clinical tests.

According to Ferenczy's investigations, bactericidal substances are most often found in Compositae and are completely absent in Gramineae. Young leaves contain more antibiotic than old leaves, and leaves contain more than stems. The detection of bactericidal substances in various parts of different plants is carried out by conventional microbiological methods (diffusion through agar blocks and velvet print method). Tests are being made on gram-positive and gram-negative bacteria and on yeasts.

In addition to this, synthetic inhibitors and their effect on fungi are being tested (J. Zsolt).

Also of great interest to microbiologists are the biochemical investigations being conducted in the Institute of Organic Chemistry (Director: F. B. Straub) of Budapest Medical University.

The general trend of research in this department is connected with the most topical problem of modern biology — the mechanism of protein synthesis.

As a working hypothesis for organizing a program of experimental research on this problem, Academician Straub has advanced the view that compounds of native specific ribonucleic acid (RNA) with an appropriate protein can reproduce themselves. The grounds for such a hypothesis and its experimental confirmation are, on one hand, the results of several recent investigations which have shown that RNA is not a chemically uniform substance, but is a mixture of nucleic acids of varying chemical structure, and this in turn substantiates the hypothesis that the number of specific nucleic acids corresponds exactly to the number of existing proteins. On the other hand, in his own earlier investigations Straub succeeded in selecting the right conditions for synthesis of pancreatic amylase. In this case, where the cell structures were destroyed, i.e., where there were no interfering side reactions, an increased synthesis of enzyme, sometimes 30-40% more than the initial, became possible.

When the protein nature of enzymes was established, the obtention of specific protein bodies became possible. Enzymatic proteins are suitable for research: first, because their quantity is easily determined by measurement of the catalytic activity of the enzyme; second, because there are well developed methods for isolation of enzymes; and third, it is quite certain that the measured rate of formation and breakdown of an enzymatic

protein actually indicates the formation and breakdown of a specific kind of protein.

However, for theoretical research on the mechanism of protein formation adult organisms are not quite suitable, since the amount of protein matter which they contain does not increase. Only objects in which a large amount of protein is formed within a short period are of interest. This is why, of course, so much attention is being given at present to the formation of the so-called adaptive enzymes.

It is of interest to mention the studies of Kramer, a colleague of Straub's. These are concerned with the deciphering of the synthesis of penicillinase in Bacillus cereus. To begin with, Kramer found that the formation of penicillinase in B. cereus could occur not only when penicillin was added to the medium, but also when RNA was added, provided that the RNA was obtained from this same species of bacterium. RNA from Escherichia coli and other material had no effect on this phenomenon. Further studies showed that, contrary to theoretical reasoning, the labels attached to the prepared RNA (to the phosphorus) from B. cereus were found, after an appropriate incubation, not in the intracellular RNA compounds nor in the acid-soluble phosphorus compounds, but in the deoxyribonucleic acid (DNA), i.e., the role of RNA in this case is still not clear — can it transmit information through DNA? The literature at present only contains information indicating that RNA is necessary for protein synthesis. The interpretation of the obtained results is made even more difficult by the fact that this effect is not hereditarily transmitted. These results have naturally aroused the interest of biologists in various fields.

Microbiological material is also being used in several other studies in the Institute. For instance, Denes is engaged in an elucidation of the factors regulating the amount of enzyme in microorganisms. It has been shown that the level of constitutive enzymes is controlled by enzymatic repression, consisting in the cessation of synthesis of a particular enzyme when particular compounds are introduced into the enzymatic systems.

The Institute's research program also includes questions of the synthesis of induced enzymes with the aim of preventing their inactivation. Work has been carried out on E. coli mutants which produce β -galactosidase. The induced strains are very sensitive and hence enzyme production in them, as distinct from noninduced strains, is inhibited by glucose.

Straub has now been appointed director of the Institute of Chemistry and Biochemistry of the Hungarian Academy of Sciences, where he is working on the problem of the relation between chemical structure and biological function of protein and enzyme. Preparations of phosphoglyceraldehyde dehydrogenase have been isolated from the muscles of various animals and from certain species of yeasts.

A. Kramli, Director of the Institute of Chemistry and Biochemistry of Szeged Medical University, is devoted

ing considerable attention to a study of the laws of oxidation relations existing in living cells of microorganisms and in the tissues of higher plants. In particular, he has studied the change in oxidation-reduction potential in microorganisms which produce streptomycin, aureomycin, riboflavin, or ergosterol. He has shown that the oxidation-reduction potential curve consists of three phases — growth, production and rest. In his opinion, it is important for industrial purposes to attempt to prolong the production phase by a choice of the corresponding conditions for the manufacture of antibiotics or other practically valuable products. For instance, Kramli succeeded in obtaining an increased yield of riboflavin by mixing old cultures of *Eremotecium ashbyi* with young ones which had not started to produce and to lower the oxidation-reduction potential. The production phase in this case was reduced by one to two days and the increase in riboflavin yield was 20%. Experiments with yeasts showed that if hemin C is bound at the right time with a certain protein complex of the yeasts there is a pronounced drop in potential and an increase in the endogenous respiration of the yeasts. The ergosterol content of yeasts treated with hemin C (2.5-10 $\mu\text{g/ml}$) in tests increased by 50-70%. Kramli believes that a determination of the oxidation-reduction potential curve in any microbiological or biochemical process should precede the laborious and often fruitless biochemical analyses. Measurements of the oxidation-reduction potential often indicate ways of selecting fermentation conditions.

In the Institute of Organic Chemistry (Director: O. Kovacs) of Szeged University, B. Matkovics is collaborating with the staff of the Institute of Plant Physiology (Director: Szalai) in a study of the intricate mechanism of assimilation of atmospheric nitrogen by means of a specific microorganism isolated by D. Nemes. Studies

by Matkovics on the effect of sterols on the growth of microorganisms are also of interest. It was shown that cholesterol and ergosterol, depending on the concentration, cause an increase or decrease in the dry weight of mycelium. The mechanism of this process is still obscure. It is possible that the addition of cholesterol to the medium increases its surface tension and thus affects the oxygen conditions of the medium and, possibly, dehydrogenase activity. Matkovics is also collaborating with the staff of the Institute of Plant Physiology in research on carotenoids in yeasts and an elucidation of the conditions of their formation and accumulation.

In the Institute of Genetics (Director: Gyorffy) there are sections dealing specially with the biochemical genetics of microorganisms. For instance, Sik is studying nucleic acids in antibiotic-resistant and susceptible strains of *Serratia marcescens*. It has been found that the ratios of the bases in the two mutants are the same. In the resistant strain slightly more guanine was found, and the aspartic acid was more labile. It is proposed to conduct research on the synthesis of nucleic acids by means of enzyme systems.

The director of the Botanical Garden of the Institute of Plant Systematics of Budapest University is R. Soo. Geobotany and the taxonomy of lower and higher plants are the main concern of this Institute. Various phytocoenoses in Hungary, including forest vegetation, meadow, rocks, cultivated plants, yeasts, and algae are being studied and mapped.

A general account of the information obtained will be published in a series of works, consisting of about 100 volumes.

In conclusion we must mention that we were warmly received in all the Institutes and that we found great friendliness toward the Soviet Union and interest in the scientific research being conducted in our country.

DEFENSE OF DISSERTATIONS

A. E. Kosmachev

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In M. V. Lomonosov Moscow State University on 18 November, 1960, E. L. Ruban defended his dissertation for the Degree of Doctor of Biological Sciences on the theme "Physiology and biochemistry of nitrifying microorganisms".

It was shown that a *Nitrosomonas* culture isolated by the drop method had a definite life cycle consisting in the alternation of a motile and nonmotile stage. In the motile stage the investigated strain was a lophotrich.

A comparative study of pure and mixed cultures showed that the associated microflora was not accidental, but was a stable complex of microorganisms. Representatives of the genera *Pseudomonas* and *Mycobacterium* stimulated nitrification, whereas sporeforming soil bacteria and myxobacteria suppressed this process. The positive effect of the associated microorganisms had no connection with vitamins, since *Nitrosomonas* independently synthesizes all the vitamins it requires. A study of the biochemical composition of *Nitrosomonas* cells confirmed the previously existing data indicating that the proteins of this microorganism contain the same amino acids as the proteins of heterotrophic bacteria, and that these microorganisms lack glucose and contain small amounts of xylose, ribose, and rhamnose. In nucleic acid composition *Nitrosomonas* differs from *Pseudomonas* and photosynthetic bacteria. The biochemical composition of *Nitrosomonas* cells changes with the age of the culture — there is a decrease in total nitrogen and a relative increase in the amount of carbon. *Nitrosomonas* ash contains considerable amounts of iron and phosphorus.

A study of the behavior of *Nitrosomonas* toward organic substances by the method of chemical analysis confirmed the existing information which indicates that *Nitrosomonas* is incapable of using sugar and organic acids. However, for the obtention of more precise data the labeled atom method was used. Comparative experiments showed that the biomass of *Nitrosomonas* acquired high activity in the presence of labeled carbonate and very low activity in the presence of labeled sucrose, whereas *Pseudomonas* gave exactly opposite results, i.e., high activity on sucrose and very low activity on sodium carbonate.

Labeled carbon of carbonate was rapidly and actively assimilated by *Nitrosomonas* cells. High concentrations

of sugars had no bactericidal action on *Nitrosomonas* and after a long period in sugar solutions this microorganism still remained active.

A study of the nitrogen metabolism of *Nitrosomonas* established that nitrification could take place, though at different rates, in cultures containing various ammonium salts. Nitrites could be produced from certain purine derivatives — guanine, uric acid, allantoin, and urea. In the case of heterotrophic microorganisms, purine derivatives are a source of carbon nutrition or are assimilated as component parts of nucleic acids. In contrast to heterotrophs, *Nitrosomonas* uses purine amino groups as an oxidizable substrate. The organic skeletons of purines are not utilized.

Not all the amino groups of purine derivatives, however, are subject to nitrification. In guanine one amino group is nitrified, in uric acid two groups, and in allantoin all the groups.

When *Pseudomonas* is present in the culture all the nitrogen-containing groups are completely nitrified. Xanthine and hypoxanthine do not undergo nitrification in pure *Nitrosomonas* cultures, but are completely nitrified by mixed cultures.

Nitrification of purine derivatives can explain the occurrence of nitrifiers in manured soils and in guano beds, which are rich in purine derivatives, and can also explain the role of the associated microorganisms, which liberate the bound amino groups of the purines.

It was found that nitrifying microorganisms were capable of storing underoxidized forms of nitrogen and using them in the absence of reducing nitrogen in the medium, i.e., of effecting "residual nitrification."

A study of cell-free nitrification showed that this process was of an enzymatic nature, i.e., it was suppressed by heating and enzyme poisons. The postulated existence of intermediate forms of underoxidized nitrogen was confirmed by the ability of cell-free autolysates to oxidize hydroxylamine to nitrites.

It was shown that the enzyme complex involved in the oxidation of ammonia to nitrites was closely bound with cell structures and that only a very slight amount came out in solution. A study of other enzyme complexes of *Nitrosomonas* showed that cells of this microorganism possess a very weak catalase and dehydrogenase activity and a high peroxidase activity. In enzyme ac-

tivity, nitrifiers differ considerably from heterotrophic organisms, which usually possess active dehydrogenases and catalase and a very weak peroxidase. The large amount of iron in *Nitrosomonas* cells, and the presence of cytochromes and peroxidase in them, shows that the oxidative process in this organism involves cytochromes, possibly flavoproteins, and that the final link in this chain is peroxidase.

Acetone preparations of nitrifying bacteria gave an intense reaction with gualacium and affected nitrification in the presence of hydrogen peroxide. Oxidation was suppressed by the addition of catalase.

In the M. V. Lomonosov Moscow State University on 9 December, 1960, P. A. Maksimova defended her dissertation for the Degree of Candidate of Biological Science on the theme "Fumagillin and the conditions of its formation."

From a collection of *Aspergillus fumigatus* Fres., the strain 4/3 was selected for its very high antiphage activity. This strain was used for studies aimed at developing methods for the industrial production of fumagillin.

A study was made of the development of strain 4/3 in submerged culture. The development of the mycelium revealed the regular features typical of other molds, in particular the penicillin producer.

It was found that the development of the antibiotic producer involved two stages, viz., growth of vegetative mycelium and then growth of productive mycelium capable of forming fumagillin. Nitrogen and carbon nutrition were studied. The greatest production of fumagil-

lin was observed when cornsteep liquor was used as a source of organic nitrogen, and the carbon source was of a slowly assimilable type, such as starch or maltose. From these investigations an industrial medium for fumagillin production was developed. This consisted of cornsteep liquor (2%) and starch (2%). Observations on the development of the producer and the increase in activity showed that nitrogen was necessary mainly for the development of the fungus in the first period of growth (nonproductive mycelium).

A nitrogen deficiency in the period of development of the productive mycelium had much less effect on its development than a carbon deficiency. At the same time it was found that the presence of carbon was absolutely essential throughout the period of fermentation. The productive mycelium, capable of the biosynthesis of the antibiotic, can develop only when carbon is present. Organic acids were shown to have no stimulating effect on fumagillin production.

The author believes that biosynthesis of fumagillin molecules in this case does not involve fragments of acetic acid. A specific stimulating effect on fumagillin production was found in the case of glycerol, glycerophosphate, and also sodium fluoride, which leads to an accumulation of glycerophosphate in the cell.

Glycerol is included in the composition of the regulation medium for fumagillin production. The results of all this work have provided a basis for the establishment of laboratory, and then pilot plant, regulations for the industrial production of fumagillin.

SERGEI IVANOVICH KUZNETSOV

(On his sixtieth birthday)

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S. I. Kuznetsov was born in 1900 in the family of the well-known Moscow architect I. S. Kuznetsov. After finishing at the Real School in 1919, S. I. Kuznetsov entered the Biological Department of the Mathematics and Physics Faculty of Moscow University. In 1923, he completed his university course with plant physiology as his special subject and was appointed to the post of assistant in microbiological research in the same department. The Department of Plant Physiology of Moscow University was headed at that time by N. Krasheninnikov, a close pupil of K. A. Timiryazev, and was developing the ideas of the celebrated physiologist and Darwinist. V. N. Shaposhnikov, A. I. Oparin, and E. E. Uspenskii, pupils and followers of K. A. Timiryazev, were working as young docents in the department. E. E. Uspenskii was interested in questions of the biochemistry and physiology of microorganisms and from 1916 he conducted an independent course on microbiology in the Department of Plant Physiology.

In 1925 microbiology became an independent department; E. E. Uspenskii was head of the department until 1938. The direction of Uspenskii's interests and his approach to the study of the vital activity of microorganisms in waters and in soil were similar to Kuznetsov's interests, which had already been decided in his student years.

In 1920, when he was a student in the second course, Kuznetsov began to work as a bacteriologist and chemist at the hydrobiological station on Lake Glubokoe. His first scientific work was devoted to physicochemical studies of Lake Glubokoe in relation to plankton distribution and was published just when he completed his university course.

Simultaneously with his work on Lake Glubokoe, Kuznetsov bore a heavy teaching burden as assistant in the Department of Microbiology of Moscow University.

During subsequent years, Kuznetsov's main investigations were directed toward a study of the role of microbiological processes in waters. A general account of these extensive investigations, which Kuznetsov carried out on the limnological stations on Lake Glubokoe and in Kosino, in the Department of Microbiology of Moscow University, and from 1942 in the Institute of Microbiology AN SSSR, as well as in numerous expeditions connected

with the study of Siberia, Karelia, and the central regions of the USSR, was given in his book "Role of Microorganisms in the Cycle of Matter in Lakes" (1950). As regards coverage of the main phenomena occurring in waters, the logical sequence of exposition of the material and the originality of approach and diversity of methods used in the study of microbiological processes in waters, Kuznetsov's monograph is a very valuable reference book on aquatic microbiology. Each chapter of this book contains not only an excellent review of the literature, but also an account of the interesting facts obtained on many topics by the author himself or his colleagues. Kuznetsov is a leading specialist in the field of aquatic microbiology and more than 50 of his works have dealt with different questions of the microbiology of waters.

A number of his works have been devoted to the development of methods for physicochemical investigations of waters. The total number of his works is about 100.

Kuznetsov made a considerable contribution to the study of soil microbiology. Working in the Department of Soil Microbiology of the Scientific Institute for Fertilizers, Kuznetsov studied the conditions of development of microbiological processes in irrigated soils of Transcaucasia and Central Asia, the conditions of preservation of organic fertilizers, and several other problems.

Special questions of the physiology of microorganisms involved in the cycle of nitrogen, carbon and sulfur in the biosphere have also attracted Kuznetsov's attention.

In recent years the research work of Kuznetsov has revealed a new trend, one which has developed rapidly in recent years — the study of the role of microorganisms in geochemical processes occurring in mineral deposits. The first studies which he and V. A. Ékzertsov conducted along this line enabled them to reveal the role of the microflora of petroleum deposits in the formation of fuel gas from petroleum. With the active participation of Sergei Ivanovich, progress has been made in several laboratories in research on the development of microbiological methods of prospecting for gas and petroleum deposits.

In the last six years a whole cycle of studies devoted to a discovery of the physiology and geochemical

role of microorganisms in the sulfur cycle has been conducted under the leadership and with the direct participation of Sergei Ivanovich. He has made a thorough study of the occurrence and role of various representatives of the genus *Thiobacillus* in many deposits of petroleum, sulfur, and sulfide ores.

As a result of Kuznetsov's efforts a new branch of microbiology — geological microbiology — the first chapters of which were written by our outstanding microbiologists, S. N. Winogradsky, G. A. Nadson, V. L. Omelian-sky, and B. L. Isachenko, has gradually amassed new facts and has assumed the rank of an independent discipline. Kuznetsov has given an account of the first scientific and practical results of this science in a monograph, "Principles of Geological Microbiology," which will be published soon.

S. I. Kuznetsov has always been eager to pass on his knowledge and experience to his research colleagues and

university students. From 1938 Sergei Ivanovich has delivered a course of lectures on aquatic microbiology in the Department of Microbiology of Moscow University. The teaching of this subject has enabled Kuznetsov to interest talented young microbiologists in questions of aquatic microbiology, and in collaboration with him they are developing this field of microbiology, which is of such importance for the economy of our country.

Kuznetsov's works have received wide recognition among our own and foreign microbiologists and limnologists. In 1944 he was awarded the degree of Doctor of Biological Sciences for his investigations on oxygen conditions in lakes. In 1960 he was elected Corresponding Member of the Academy of Sciences, USSR, for his great services in the development of microbiological science.

We wish S. I. Kuznetsov further successes in his many-sided and fruitful work.

NIKOLAI DMITRIEVICH IERUSALIMSKII

(On his sixtieth birthday)

Translated from *Mikrobiologiya*, Vol. 30, No. 2,
p. 381, March-April, 1961

On 17 January 1961, Nikolai Dmitrievich Ierusalimskii, Doctor of Biological Sciences and Corresponding Member of the Academy of Sciences, USSR, celebrated his 60th birthday and the completion of 30 years of scientific, teaching, and public work.

The scientific vocation of N. D. Ierusalimskii was decided while he was still in his student years. After his normal evening studies he was then devoting his time to microbiological investigations. He made a study of the distribution of the bacterial population of Moscow River and its tributaries. This work was published in the journal *"Mikrobiologiya"* in 1932. In his final course at Moscow State University, which he completed in 1931, Ierusalimskii was attracted by the new industrial microbiology section which had just been set up by V. N. Shaposhnikov. This determined the direction of all his future work.

Ierusalimskii's scientific research after he had finished university was devoted to the acetone-butanol fermentation. He took a direct part in the organization of the first acetone plant in the Soviet Union in Grozny (1934).

At that time Ierusalimskii was already interested in the laws of development of microorganisms. Whatever problems confronted him in the study of various fermentations, he always made a thorough study of the physiological stages of development of the microorganisms. For instance, in the successful tackling of the problem of increasing the output of an operating vinegar plant, Ierusalimskii made a thorough study of the physiology of growth of the microorganisms involved.

Several important investigations of Nikolai Dmitrievich were concerned with the acetone-ethanol, lactic acid, and butyric acid fermentations. In these works he successfully resolved various problems — choice of media and raw material, dependence of fermentation on nature of nitrogen nutrition, and the vitamin requirements of bacteria. He never failed to study growth and physiology, and also the physiological stages of development.

In his doctoral dissertation Ierusalimskii mainly summed up the results of his many years of work on the laws of bacterial growth and development.

In many works Ierusalimskii studied the nitrogen and vitamin nutrition of microbes. On this subject he compiled a special course of lectures, which he delivered for 15 years to students of Moscow State University. In Nikolai Dmitrievich's book, *"Nitrogen and Vitamin Nutrition of Microbes,"* he summed up his own investigations

and also gave a critical review of the research carried out in this field.

In the years when the antibiotics industry was being developed, Ierusalimskii and his colleagues studied the physiology of the penicillin-producing fungi.

Of particular value are Ierusalimskii's works on the continuous cultivation of microorganisms. By means of this method he is studying spore formation in bacteria and the physiology of various producers of vitamin B₁₂. We must mention that he has designed original laboratory apparatus for the continuous cultivation of microorganisms.

By the continuous cultivation method, Ierusalimskii has shown and substantiated theoretically that in a medium in which the usual conditions appropriate to the requirements of the particular microorganisms prevail, the microorganisms remain indefinitely in the same physiological state. In unusual conditions, particularly in the presence of increasing doses of poisonous compounds, their metabolism is altered accordingly, and in time this alteration assumes a hereditary nature.

Ierusalimskii was the initiator and organizer of the First All-Union Conference on Continuous Fermentation and Cultivation of Microorganisms.

About 90 scientific works by Ierusalimskii have been published. He enjoys well-deserved prestige both in the Soviet Union and abroad. Ierusalimskii has several times delivered scientific papers at international symposia and congresses on questions of adaptation, continuous cultivation of microbes, microbial physiology, and so on. Ierusalimskii is conducting great social and political and scientific administration work. For more than ten years he has worked as Deputy Director of the Institute of Microbiology, AN SSSR, and is a member of the Party bureau. He is the representative of the USSR on COSPAR (International Committee on Space Research), a member of the editorial board of the journal *"Mikrobiologiya,"* and a member of the Export Commission VAK on biology.

Endowed with great knowledge, Ierusalimskii is eagerly helping many scientific institutions and industries, as well as his colleagues and, in particular, young specialists.

For his great scientific and teaching work, Ierusalimskii has been awarded two "Badge of Honor" orders and medals.

On his sixtieth birthday, we wish N. D. Ierusalimskii further constructive achievements in his many spheres of work.



SERGEI NIKOLAEVICH MUROMTSEV
(1898-1960)

On 14 December, 1960, Sergei Nikolaevich Muromtsev, Director of the Gamaleya Institute of Epidemiology, AMN SSSR, Academician of the All-Union Academy of Agricultural Sciences (VASKhNIL), Doctor of Biological Sciences, Stalin Prize winner, and member of the C.P. S.U., died after a brief illness.

Muromtsev was born on 26 May, 1898, into a teacher's family in the village of Makkaveevo in Kasimov District, Ryazan Province. In 1923 he finished at the Medical Faculty of Moscow University and in the same year he completed a special course in the Red Army Academy of Chemical Defense.

Muromtsev had a great career. He was a scientific worker in Bukhara Tropical Institute, and in the I. I. Mechnikov Moscow Regional Institute of Infectious Diseases (1925-1928). Muromtsev was one of the organizers and directors of the control of veterinary biopreparations in the country.

From 1934 to 1937 Sergei Nikolaevich directed the microbiology laboratories in the All-Union Institute of Veterinary Medicine and in the Erisman Moscow Sanitary Institute, and from 1937 he directed a laboratory in the MVD, from which he transferred in 1951 to a permanent post in VASKhNIL, where he had held an additional post since 1939.

Muromtsev directed the Laboratory of Microbiology and Virology in VASKhNIL until 1956, and in March of

that year he became head of the N. F. Gamaleya Institute of Epidemiology and Microbiology (IEM), the largest institute of this kind in our country. Death overtook him in the midst of administrative and constructive activity. During his office several new laboratories were set up under his guidance in the IEM — the Laboratory of Fluorescent and Electron Microscopy, the Laboratory for the Production of Diagnostic Fluorescent Sera, and the Aerosol Laboratory. The last was a pet child of Muromtsev; he was not only its organizer, but also its scientific director.

Muromtsev's pen has been responsible for more than 100 works, including seven monographs. The range of his constructive scientific interests was very wide, and he made a considerable contribution to the resolution of important questions of general, medical, and agricultural microbiology. He was always particularly interested in the problem of microbial variation, which he developed consistently from the standpoint of Michurinist biology.

A general account of Muromtsev's numerous investigations on this immense problem has been given in his monographs: "Problems of Modern Biology in the Light of Michurinist Teaching" (1950), "Variation of Microorganisms and Problems of Immunity" (1946). These works, and also the paper, "The species problem in microbiology" [Agrobiologiya, 2 (1952)], contain many original views, which characterize the deceased scientist

as a singular research worker with a wide range of thought. In these works he expressed several ideas on the possibility of the transformation of saprophytic forms into pathogenic forms, the nature of vaccine strains, and the epidemiology of certain infections of men and animals.

One of his recent papers was "Microorganisms as objects for the study of some general biological questions" (Zhur. Mikrobiol., Epidemiol. i. Immunobiol., 2, 1952, and the collection: "Problems of Epidemiology and Microbiology" [in Russian], Part 1, 1959).

Research in the field of microbial variation and heredity enabled Muromtsev to put forward a number of important theoretical and practical suggestions. For instance, his suggestion in 1929 of a killed vaccine against emphysematous carbuncle of cattle and a curative serum have been adopted in practice. His colleagues and pupils have developed vaccines against several other anaerobic diseases.

Between 1934 and 1943 he developed and introduced into practice a new principle in the preparation of effective vaccines — the so-called semiliquid vaccines, which are being used against a number of infectious diseases of farm animals. The studies of Muromtsev and his colleagues on this problem were summed up in the monograph "Semiliquid Vaccines" (1944), which was awarded a Stalin Prize in 1945. His services in the control of diseases of farm animals were recognized by the award of a Gold Medal of the All-Union Agricultural Exhibition.

Muromtsev has also done considerable work on certain other questions of microbiology. For instance, a

number of his works have been devoted to phage prophylaxis and phage therapy of infectious diseases of man and animals, and to the study of rabies and certain other infections. In particular, we should mention that in 1926 he proposed a method, now adopted in practice, for rapid diagnosis of rabies by special fixation and staining of smears. These investigations were valuable for revealing the structure of Negri bodies and, hence, the nature of the rabies agent.

Muromtsev performed great social work as a member of the board of the I. I. Mechnikov All-Union Society of Microbiologists, Epidemiologists, and Infectionists, member of the Expert Commission of the Lenin Prize Committee, President of the Biological Section of the All-Union Society for the Dissemination of Political and Scientific Knowledge, member of the Biological Section of the Union of Societies for Cultural Relations with Foreign Countries, member of the Soviet of the House of Scientists, AN SSSR. He was elected an Honorary Member of the International Committee on Vegetative Hybridization in Geneva (1960).

Muromtsev's fruitful scientific and public work has been acknowledged by many State awards: Order of the Labor Red Banner, two orders of the Red Banner, two orders of the Red Star and the medals "For War Service," "Partisan of the Patriotic War," "For the Defense of Moscow." The name of S. N. Muromtsev will live in the history of microbiology as that of a progressive scholar and a talented organizer of science.

Yu. I. Milenushkin

Translated from Mikrobiologiya, Vol. 30,
No. 2, pp. 382-383, March-April, 1961

ERRATA

Vol. 30, No. 1

Page	Location	Reads	Should read
170	Line 42, second word	systemotology	systematology
170	Last paragraph, first sentence	tent of the most important information and facts.	The completeness of the abstract is of substantial significance. By this I mean, not its length, but its content of the most important information and facts.
177	Fifth paragraph, first sentence	Under these conditions it is necessary to add either ergos- terol or a Tween 80 prepration containing ergosterol to the medium.	Under these conditions it is necessary to add either ergosterol or a Tween 80 prep- aration containing oleic acid to the medium.

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED
IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for Experimental Morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

Soviet Journals Available in Cover-to-Cover Translation

ABBREVIATION	RUSSIAN TITLE	TITLE OF TRANSLATION	PUBLISHER	TRANSLATION BEGAN
			Vol. Issue Year	
AĖ	Atomnaya énergiya	Soviet Journal of Atomic Energy	Consultants Bureau	1 1956
Akust. zh.	Akusticheskii zhurnal	Soviet Physics - Acoustics	American Institute of Physics	1 1955
	Antibiotiki	Antibiotics	Consultants Bureau	4 1959
Astr(om). zh(urn).	Astronimicheskii zhurnal	Soviet Astronomy—AJ	American Institute of Physics	1 1957
Avto(mat). svarka	Avtomaticheskaya svarka	Automatic Welding	British Welding Research Association (London)	1 1959
	Avtomatika i Telemekhanika	Automation and Remote Control	Instrument Society of America	27 1 1956
	Biofizika	Biophysics	National Institutes of Health*	1 1957
Byull. éksp(erim). biol. i med.	Biokhimiya	Bulletin of Experimental Biology and Medicine	Consultants Bureau	21 1 1956
DAN (SSSR)	Byulleten' éksperimental'noi biologii i meditsiny		Consultants Bureau	41 1 1959
Dokl(ady) AN SSSR	Doklady Akademii Nauk SSSR	The translation of this journal is published in sections, as follows:		
		Doklady Biochemistry Section	American Institute of Biological Sciences	106 1 1956
		Doklady Biological Sciences Sections (Includes: Anatomy, biophysics, cytology, ecology, embryology, endocrinology, evolutionary morphology, genetics, histology, hydrobiology, microbiology, morphology, parasitology, physiology, zoology sections)	American Institute of Biological Sciences	112 1 1957
		Doklady Botanical Sciences Sections (Includes: Botany, phytopathology, plant anatomy, plant ecology, plant embryology, plant physiology, plant morphology sections)		
		Proceedings of the Academy of Sciences of the USSR, Section: Chemical Technology	Consultants Bureau	106 1 1956
		Proceedings of the Academy of Sciences of the USSR, Section: Chemistry	Consultants Bureau	106 1 1956
		Proceedings of the Academy of Sciences of the USSR, Section: Physical Chemistry	Consultants Bureau	112 1 1957
		Doklady Earth Sciences Sections (Includes: Geochemistry, geology, geophysics, hydrogeology, mineralogy, paleontology, petrography, permafrost sections)		
		Proceedings of the Academy of Sciences of the USSR, Section: Geochemistry	American Geological Institute	124 1 1959
		Proceedings of the Academy of Sciences of the USSR, Section: Geology	Consultants Bureau	106- 1 1957- 1958
		Proceedings of the Academy of Sciences of the USSR, Sections: Geology	Consultants Bureau	123 6 1957- 1958
		Doklady Soviet Mathematics	The American Mathematics Society	131 1 1961
		Soviet Physics—Doklady (Includes: Aerodynamics, astronomy, crystallography, cybernetics and control theory, electrical engineering, energetics, fluid mechanics, heat engineering, hydraulics, mathematical physics, mechanics, physics, technical physics, theory of elasticity sections)		
		Proceedings of the Academy of Sciences of the USSR, Applied Physics Sections (does not include mathematical physics or physics sections)	American Institute of Physics	106 1 1956
		Wood Processing Industry		
		Telecommunications	Consultants Bureau	106- 1 1956- 1957
		Entomological Review	Timber Development Association (London)	9 1959
		Pharmacology and Toxicology	Massachusetts Institute of Technology*	1 1957
		Physics of Metals and Metallography	American Institute of Biological Sciences	38 1 1959
		Sechenov Physiological Journal USSR	Consultants Bureau	20 1 1957
		Plant Physiology	Acta Metallurgica*	5 1 1957
		Geochemistry		
		Soviet Physics—Solid State	National Institutes of Health*	1 1957
		Measurement Techniques	American Institute of Biological Sciences	4 1 1957
		Bulletin of the Academy of Sciences of the USSR: Division of Chemical Sciences	The Geochemical Society	1 1958
			American Institute of Physics	1 1959
			Instrument Society of America	1 1959
			Consultants Bureau	1 1952

Izv. AN SSSR, O(td), T(ekhn), N(auk): Met(ali), i top.	(see Met, i top.) Izvestiya Akademii Nauk SSSR: Seriya fizicheskaya	Bulletin of the Academy of Sciences of the USSR: Physical Series	Columbia Technical Translations	1	1954
Izv. AN SSSR Ser. fiz(ich).	Izvestiya Akademii Nauk SSSR: Seriya geofizicheskaya	Sciences USSR: Geophysics Series	American Geophysical Union	1	1954
Izv. AN SSSR Ser. geofiz.	Izvestiya Akademii Nauk SSSR: Seriya geologicheskaya	Izvestiya of the Academy of Sciences of the USSR: Geologic Series	American Geological Institute	1	1958
Izv. AN SSSR Ser. geol.	Kauchuk i rezina	Soviet Rubber Technology	Research Association of British Rubber Manufacturers	18	1959
Kauch. i rez.	Kinetika i kataliz Koks i Khimiya	Kinetics and Catalysis Coke and Chemistry USSR	Consultants Bureau (Leeds, England)	1	1959
	Kolloidnyi zhurnal	Colloid Journal	Coal Tar Research Association	3	1960
Kolloidn. zh(urn).	Kristallografiya	Soviet Physics - Crystallography	American Institute of Physics	1	1958
Metalov. i term. obrabot. metal.	Metallovedenie i termicheskaya obrabotka metallov	Metal Science and Heat Treatment of Metals	Consultants Bureau	2	1952
Met. i top. Mikrobiol. OS	Metallurgiya i topliva Mikrobiologiya Optika i spektroskopiya Pochovovedenie Priborostroenie	Russian Metallurgy and Fuels Microbiology Optics and Spectroscopy Soviet Soil Science Instrument Construction	American Institute of Physics	1	1957
Pribory i tekhn. éks(perimenta)	Pribory i tekhnika éksperimenta	Instruments and Experimental Techniques	Acta Metallurgica	6	1958
Prikl. matem. i mekh.	Prikladnaya matematika i mekhanika	Applied Mathematics and Mechanics	Acta Metallurgica	1	1957
PTÉ	(see Pribory i tekhn. éks.)		Eagle Technical Publications	1	1960
Radiotekh.	Problemy Severa	Problems of the North	American Institute of Biological Sciences	26	1957
Radiotekh. i élektronika	Radiotekhnika i élektronika Stanki i instrument Stal'	Radio Engineering and Electronics Machines and Tooling Stal (in English)	American Institute of Physics	6	1959
Stek. i keram.	Svarochnoe proizvodstvo	Glass and Ceramics Welding Production	British Scientific Instrument Research Association	1	1958
Svaroch. proizvo	Teoriya veroyatnostei i ee primeneniye	Theory of Probability and Its Applications	Instrument Society of America	1	1959
Teor. veroyat. i prim.			American Society of Mechanical Engineers	1	1957
Tsvet. Metall	Tsvetnye metally	Nonferrous Metals		1	1958
UFN	Uspekhi fizicheskikh Nauk	Soviet Physics - Uspekhi (partial translation)	National Research Council of Canada	1	1957
UKh	Uspekhi khimii	Russian Chemical Reviews	Massachusetts Institute of Technology*	12	1957
UMN	Uspekhi matematicheskikh nauk	Russian Mathematical Surveys	Production Engineering Research Assoc.	2	1957
Usp. fiz. nauk	(see UFN)		Iron and Steel Institute	1	1959
Usp. khim(ii)	(see UKh)		Consultants Bureau	1	1956
Usp. matem. nauk	(see UMN)		British Welding Research Association	4	1959
Usp. sovr. biol.	Uspekhi sovremennoi biologii		Society for Industrial and Applied Mathematics	1	1956
Vest. mashinostroeniya	Vestnik mashinostroeniya		Primary Sources	1	1960
Vop. gem. i per. krovi	Voprosy gematologii i pereivaniya krovi		American Institute of Physics	66	1958
Vop. onk.	Voprosy onkologii		The Chemical Society (London)	1	1960
Vop. virusol.	Voprosy virusologii		London Mathematical Society	15	
Zav(odsk), lab(oratoriya)	Zavodskaya laboratoriya		Oliver and Boyd	48	1959
ZhAKh Zh. anal(it), khimii	Zhurnal analiticheskoi khimii		Production Engineering Research Assoc.	4	1959
ZhETF	Zhurnal éksperimental'noi i theoreticheskoi fiziki		National Institutes of Health*	1	1957
Zh. éksperim. i teor. fiz.	Zhurnal fizicheskoi khimii		National Institutes of Health*	1	1957
ZhFKh Zh. fiz. khimii	Zhurnal fizicheskoi khimii		Instrument Society of America	25	1957
ZhMEI Zh(urn), mikrobiol. épidemiol. i immunobiol.	Zhurnal mikrobiologii, épidemiologii i immunobiologii		Consultants Bureau	7	1952
ZhNKh	Zhurnal neorganicheskoi khimii		American Institute of Physics	28	1955
Zh(urn), neorgan(ich). khim(ii)			The Chemical Society (London)	7	1959
ZhOKh	Zhurnal obshchei khimii		National Institutes of Health*	1	1957
Zh(urn), obshch(ei) khimii			National Institutes of Health*	1	1957
ZhPKh	Zhurnal prikladnoi khimii		Instrument Society of America	25	1957
Zh(urn), prikl. khimii			Consultants Bureau	7	1952
ZhSKh	Zhurnal strukturnoi khimii		The Chemical Society (London)	1	1957
Zh(urn), strukt. khimii			Consultants Bureau	19	1949
ZhTF	Zhurnal tekhnicheskoi fiziki		Consultants Bureau	23	1950
Zh(urn), tekhn. fiz.			Consultants Bureau	1	1960
Zh(urn), vyssh. nervn. deyat. (im. P. Pavlova)	Zhurnal vysshei nervnoi deyatel'nosti (im. I. P. Pavlova)		American Institute of Physics	26	1956
			National Institutes of Health*	1	1958

*Sponsoring organization. Translation through 1960 issues is a publication of Pergamon Press.

AIBS Russian Monograph Translations

The AIBS is in the process of expanding its Russian Translations Program extensively. Funds to subsidize translation and publication of important Russian literature in biology have been obtained from the National Science Foundation, as part of a larger program to encourage the exchange of scientific information between the two countries. The following monographs have been published:

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68 pp. \$3.00, individuals and industrial libraries (U.S.A. and Canada);
\$2.00, AIBS members and all other libraries; \$.50 additional, foreign.

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Problems in the Classification of Antagonists of Actinomycetes. *By G. F. Gause.*

Edited by David Gottlieb. Translated by Fritz Danga.

\$5.00, individuals and industrial libraries (U.S.A. and Canada);
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Arachnida. Vol. IV, No. 2. Fauna of the U.S.S.R. *By B. I. Pomerantzev.*

Edited by George Anastos. Translated by Alena Elbl.

\$10.00, individuals and industrial libraries (U.S.A. and Canada);
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Arachnoidea. Vol. VI, No. 1. Fauna of the U.S.S.R. *By A. A. Zachvatkin.*

Translated and edited by A. Ratcliffe and A. M. Hughes.

\$10.00, individuals and industrial libraries (U.S.A. and Canada);
\$9.00, AIBS members and all other libraries; \$1.00 additional, foreign.

Marine Biology. Vol. XX, Trudy Institute of Oceanology. *Edited by B. N. Nikitin.*

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\$7.50, AIBS members and all other libraries; \$1.00 additional, foreign.

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